XLVII. ON THE NATURE OF THE CELL WALL CONSTITUENTS OF *LAMINARIA* SPP. MANNURONIC ACID.

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The nature of the cell wall constituents of the red and brown algae has been the subject of study in this laboratory for some time past [Haas and Hill, 1921; Haas, 1921; Russell-Wells, 1922; Haas and Russell-Wells, 1923, 1929]. The present investigation, which is concerned primarily with *Laminaria* spp., was undertaken with the object of throwing some light on the mode of occurrence within the plant of some of the substances which have from time to time been described as being obtained from this genus. The search for the source of the methylpentose, fucose, in particular, has involved the study of the products of hydrolysis of two cell wall constituents known respectively as fucoidin and alginic acid and the results obtained are described herein.

Fucoidin.

The term fucoidin was first applied by Kylin [1913] to the highly mucilaginous material which exudes from freshly collected samples of *Laminaria* spp. and from *Fucus serratus*, *F. vesiculosus* and *Ascophyllum nodosum*. He found it to be a calcium salt and observed that when extracted from the weed with hot water the material was much less viscous; this fact he attributed to the conversion of the calcium salt into the alkali metal salt, though no evidence was offered for his assertion; he stated further that fucoidin contains pentoses and methylpentoses and established the presence of fucose among the products of hydrolysis of this substance.

Some years ago Haas and Russell-Wells [1923] obtained evidence of the existence of an ethereal sulphate grouping in *Laminaria*, and it has now been possible to show that this grouping is actually contained in fucoidin. The method employed for the isolation and purification of this substance was as follows.

The freshly gathered weed was soaked in distilled water whereby a very translucent and highly viscous extract was produced from which, by addition of an equal volume of absolute alcohol, a gelatinous precipitate was obtained. This substance when placed in water again swelled up and went into solution, but gradually lost its gelatinous character on keeping; it was purified by solution in water and re-precipitation by means of alcohol; but with the

gradual removal of salts, precipitation by alcohol became increasingly difficult and this method of purification was abandoned in favour of dialysis. When all dialysable salts had been removed the dialysed solution was poured into alcohol and the precipitated material was preserved in alcohol, since drying caused shrinkage to a tough horny material, which though still soluble in water had a greatly reduced swelling power.

On incineration the material yielded 30·93 % of ash, consisting for the most part of calcium sulphate. The calcium was shown to occur in the original material in the ionised condition, being precipitable by ammonium oxalate. That the substance was an ethereal sulphate was shown by the fact that the original material dissolved in water gave no reactions for the sulphate ion but that after hydrolysis it yielded precipitable sulphate in approximately double the quantity of that in the ash.

Sulphate in hydrolysed solution = 30·33 %,
ash = 15·10 %.

The presence of methylpentose, described by Kylin, was confirmed both by the colour reaction of Rosenthaler and by the isolation of an osazone crystallising in yellow needles, M.P. 170–173°, from the products of hydrolysis of the material with 3 % sulphuric acid in a boiling water-bath for 11 hours. The preparation from *Fucus* spp. of fucose was first described by Günther and Tollens [1890] and later by Votoček [1915] and by Clark [1922] who hydrolysed the entire weeds, after a preliminary soaking in dilute sulphuric acid. In view however of the evidence here presented for the occurrence of fucose in the water-soluble fucoidin of *Laminaria*, it appears that the preliminary soaking in dilute acid may remove some of the potential source of this sugar[1]. Heated with 12 % hydrochloric acid fucoidin produced furfuraldehyde which yielded a black phloroglucide soluble in alcohol; carbon dioxide was likewise given off, corresponding to about 7·3 % of a uronic acid complex, but, owing to the limited amount of material available, no attempt was made to ascertain its nature.

Algin and alginic acid.

The term algin was first applied by Stanford [1883, 1884, 1886] to a mucilaginous material occurring in *Laminaria* fronds; on extracting the weed with cold 2 % sodium carbonate and acidifying the resulting solution he obtained a precipitate which he described as alginic acid; of this acid he prepared a number of salts including an insoluble calcium salt containing 7·84 % of calcium.

Kylin [1915] applied the term algin to a supposed insoluble calcium salt which he assumed to occur in the cell wall; he stated that it was insoluble

[1] The completely exhausted residue from the extraction of 200 g. of *Laminaria* fronds with 2 % sodium carbonate, weighing 80 g., when hydrolysed under the conditions described by Clark [1922] for *Fucus vesiculosus*, yielded a very small quantity of an osazone which could not be definitely identified with that of fucose. From this fact it would appear that in the case of *Laminaria* at all events, the chief source of fucose would be the fucoidin.

or very sparingly soluble in water and suggested that hot water extracted from the weed an alkali metal salt of alginic acid produced from the calcium salt, but he did not support this by any experimental evidence. His explanation, moreover, of the action of sodium carbonate in extracting alginic acid was that it underwent double decomposition with the insoluble calcium salt yielding soluble sodium alginate.

In these circumstances it seemed worth while to examine the nature of the material extracted by hot water and to ascertain whether there were any limit to the extent to which the alginic acid contained in the cell wall could be extracted by this method. To this end 180 g. of *Laminaria* fronds were cut up, tied in a calico bag and extracted with boiling water. In view of the fact that water would also extract fucoidin, the first extracts were rejected until the absence of fucoidin could be established by hydrolysing with hydrochloric acid and showing that no sulphate was formed. The extraction was then continued until the extracts contained only negligible amounts of material; they were then evaporated to small bulk and precipitated with alcohol. The precipitated material was dissolved in a smaller volume of water and dialysed; after dialysis was complete, the material could no longer be precipitated by the addition of alcohol and was therefore recovered from solution by evaporation on a water-bath, when it weighed 2 g. Thus dried it would no longer dissolve in water. Analysis showed that it contained 3·46 % Ca and 0·45 % Mg, together with undetermined quantities of alkali metals. From these facts it was taken to be a water-soluble calcium, magnesium, sodium and potassium salt of alginic acid, since it gave, on acidification, a gelatinous precipitate in every way resembling alginic acid. That the extraction with hot water had not removed all the alginic acid from the cell wall was shown by the fact that the residue remaining, when treated with cold 2 % sodium carbonate, at once yielded an extract from which a copious precipitate of alginic acid could be obtained on acidification.

Direct evidence as to the correctness, or otherwise, of Kylin's assumption that alginic acid occurs in the cell wall in the form of an insoluble calcium salt is not easy to furnish, although the readiness with which cold dilute sodium carbonate extracts this acid from the weed is rather against this assumption. The following facts however provide indirect evidence which casts doubt on the occurrence of the calcium salt. From the loss in weight by 200 g. of the weed on exhaustive extraction with 2 % sodium carbonate it was estimated that *Laminaria* contains about 60 % of its weight of alginic acid. Such a proportion of this acid, if occurring as assumed by Kylin, in combination with calcium, would require a calcium content of the weed of 5·1 %, calculated on the basis of the figure given by Stanford for the percentage of calcium in the calcium salt. The calcium content of the whole weed was however found by analysis to be only 1·99 %.

From the above facts it is concluded that the cell wall of *Laminaria* contains alginic acid in two forms, one a soluble calcium, magnesium, alkali metal

salt, present only to the extent of about 1 %, and the other in the free state which appears to be the material extracted by cold dilute sodium carbonate; there does not thus appear to be any need to assume, as Kylin did, that the bulk of the alginic acid occurs in the form of an insoluble calcium salt.

Hydrolysis of alginic acid.

The first attempt to investigate systematically the hydrolysis of alginic acid was undertaken by Hoagland and Lieb [1915]. These authors obtaining their alginic acid from *Macrocystis pyrifera* subjected it to hydrolysis with hydrochloric acid and claimed to have obtained evidence that this substance yielded pentoses which were somewhat doubtfully identified by means of phenylhydrazine as xylose and arabinose. Some years later Schmidt and Vocke [1926] in an examination of alginic acid prepared from *Fucus serratus* described the hydrolysis of this material by successive treatments with anhydrous formic acid, 80 % sulphuric acid, and finally boiling 4·5 % sulphuric acid; they claimed to have established that alginic acid is a polymerised glycuronic acid by preparing a cinchonine salt having M.P. 204°, which is the M.P. given by Neuberg [1900] for the cinchonine salt of glycuronic acid prepared by the hydrolysis of euxanthone.

In view of these conflicting results we decided to reinvestigate the hydrolysis of alginic acid with a view to ascertaining whether it could be regarded as a potential source of pentoses or even of fucose.

For our experiments we chose sulphuric acid in preference to the hydrochloric acid employed by Hoagland and Lieb. The alginic acid[1] was heated under reflux with 8 times its weight of 2 % sulphuric acid in a boiling water-bath for 3 hours. At the end of this time the acid solution was filtered from the insoluble residue A (see below). On removing the sulphuric acid with barium carbonate it was found that the filtrate contained considerable quantities of barium in solution indicating that an acid forming a soluble barium salt had been produced.

In order to ascertain whether any hexose or pentose sugars had been produced as well, as had been suggested by Hoagland and Lieb's experiments on alginic acid from *Macrocystis pyrifera*, the barium salt in solution was precipitated by addition of alcohol. The aqueous alcoholic filtrate was filtered and evaporated to dryness under reduced pressure; the resulting light brown residue containing barium proved to be some of the barium salt which had escaped precipitation. In order however to ascertain whether it contained any sugars as well, it was repeatedly extracted with 80 % alcohol. Hardly any material was dissolved, but the combined extracts were evaporated and were found still to contain barium. Addition of phenylhydrazine acetate to the cold solution gave an immediate precipitate similar to the one described

[1] For much of the alginic acid used in these experiments we have to thank Messrs Nobel and Co., Explosives Ltd., Ardeer, Scotland.

on p. 409 and this proved to be insoluble in all organic solvents, showing that it contained no sugar hydrazones. The absence of sugars was thus established, and consequently alginic acid has to be excluded as a possible source of fucose.

Our results therefore failed to corroborate the observations of Hoagland and Lieb that alginic acid gave rise to pentoses on hydrolysis. In order to ascertain whether alginic acid from *Macrocystis pyrifera* was in any way different from that of *Laminaria* we procured some of the former weed from California through the good offices of the Californian Academy of Sciences, and on subjecting it to the same conditions of hydrolysis with sulphuric acid as described above we obtained substantially the same result, namely a solution containing a soluble barium salt. On treating this solution with phenylhydrazine as described by these authors we obtained in the cold a red-brown precipitate which contained barium but were unable to establish the presence of sugars.

In view of the statement by Schmidt and Vocke referred to above it seemed likely that the barium salt produced in our experiments might be that of glycuronic acid. On preparing the cinchonine salt by addition of the calculated amount of cinchonine sulphate to the barium salt we obtained a substance whose melting point, 195–197° (on one occasion 203°), seemed sufficiently close to that quoted by Schmidt and Vocke to suggest that it actually was cinchonine glycuronate; the specific rotation however, was $[\alpha]_D = 112{\cdot}8°$ as compared with $[\alpha]_D = 138{\cdot}6°$ as given by Neuberg. On preparing the quinine salt[1] an even greater discrepancy was found; the constants given by Neuberg for quinine glycuronate are M.P. 180° and $[\alpha]_D = -80{\cdot}1°$ and the analysis indicated the formula $C_{26}H_{34}O_9N_2$. Our salt on the other hand had M.P. 162–163° and $[\alpha]_D = -175{\cdot}3°$, while the analysis showed that the substance contained two molecules of water of crystallisation, as may be seen from the following figures[2]:

	C %	H %
Found	56·01	6·92
Calc. for $C_{26}H_{34}O_9N_2$, $2H_2O$	56·32	6·86

The residue A (p. 406) remaining after the hydrolysis of alginic acid for 3 hours with 2 % sulphuric acid was then subjected to further hydrolysis, with a view to obtaining an increased yield, by heating with 5 % sulphuric acid for 8 hours. After removal of the sulphuric acid a new cinchonine salt was obtained having M.P. 161° and $[\alpha]_D = 154°$; this substance gave the following results on analysis:

	C %	H %	N %
Found	59·21	6·87	5·4
Calc. for $C_{25}H_{32}O_5N_2$, H_2O	59·29	6·71	5·33

This salt therefore differed from that described by Schmidt and Vocke both in M.P. and in composition, since it contained a molecule of water of

[1] See Addendum.

[2] All the micro-analyses were carried out by Dr Ing. A. Schoeller of Berlin.

crystallisation. It was produced in much smaller yield than the cinchonine salt M.P. 195–197°. No explanation is at present offered for the existence of these two salts, more particularly since only one quinine salt has, so far, been obtained, but in any case neither of them agreed in physical constants with cinchonine glycuronate.

There seemed therefore no doubt that the acid produced by the hydrolysis of alginic acid from *Laminaria* was not glycuronic acid; as moreover the constants obtained did not agree for the cinchonine salt of galacturonic acid and furthermore alginic acid on oxidation yielded no trace of mucic acid, it seemed natural to conclude that the acid in question might be the hitherto unknown mannuronic acid, especially in view of the plentiful occurrence of mannitol in *Laminaria* and allied fucoids.

To settle this question experiments were undertaken[1] to ascertain the nature of the dicarboxylic acid produced on oxidation of the aldehydo-acid in question. To this end both alginic acid and its product of hydrolysis were oxidised and in each case mannosaccharic acid was identified as the diamide, thus proving conclusively that the aldehydo-acid was not glycuronic acid but the hitherto undescribed mannuronic acid.

Oxidation to mannosaccharic acid.

(*a*) *Alginic acid.* Earlier experiments on the oxidation of alginic acid by heating on a water-bath with nitric acid in search of the possible formation of mucic acid failed to produce anything but oxalic acid. By carefully regulating the oxidation however a different result was obtained.

5 g. of dried and finely ground alginic acid were covered with 7·5 g. of nitric acid (sp. gr. 1·2); the acid was at once absorbed leaving the alginic acid as a coarsely granulated mass; this was heated under a reflux condenser for 24 hours at 50°; about 50 cc. of water were now added and after thorough mixing the insoluble residue was filtered off on a Büchner funnel. The filtrate was then rapidly evaporated over a water-bath to about half its volume and then to dryness in a desiccator over sodium hydroxide, when a light lemon-yellow syrup resulted. On stirring this with concentrated ammonia it turned brown and deposited in the course of a few minutes a mass of crystals which on recrystallising from water separated in well formed rhombs melting at 188–189°.

Analysis gave the following results:

	C %	H %	N %
Found	34·39	5·84	13·21
Calc. for $C_6H_{12}O_6N_2$	34·61	5·77	13·46

[1] Experiments to this end were already in progress when we accidentally came across a paper by Cretcher and Nelson [1929], whose publication we had overlooked, in which these authors had already come to the same conclusion and had actually established the production of mannosaccharic acid on oxidation. Notwithstanding this we completed our investigation and now publish our results because they were arrived at independently and by slightly different methods.

(*b*) *Mannuronic acid.* 5·4 g. of barium mannuronate dissolved in 20 cc. of water were freed from barium by the addition of the requisite amount of dilute sulphuric acid and, after filtering, the solution was treated with 16 g. of bromine. The mixture was thoroughly shaken at intervals during 4½ days, after which the excess of bromine was removed by a rapid current of air until the liquid was of a light straw colour. The liquid was then diluted and treated with freshly precipitated silver oxide until neutral and then with hydrogen sulphide to precipitate any dissolved silver as sulphide. The filtrate evaporated to dryness in a desiccator left a colourless viscous residue which on addition of concentrated ammonia deposited rhombic prisms; these after crystallising twice from water melted at 188–189°.

Mannuronic acid.

The oxidation experiments described above showed undoubtedly that alginic acid gave rise on hydrolysis to mannuronic acid, since oxidation of the product led to mannosaccharic acid, identified by comparison of the M.P. of its diamide with that of an authentic specimen and the determination of a mixed melting point.

A sample of the mannuronic acid prepared from its barium salt left a light amber-coloured resin which did not crystallise. An attempt further to characterise the barium salt by treatment of the cold aqueous solution with phenylhydrazine acetate resulted in the production of an immediate red-brown precipitate containing barium. This material on extraction in a Soxhlet with chloroform lost its red colour and became orange-coloured. This substance was insoluble in organic solvents but dissolved sparingly in boiling water separating again rapidly on cooling in spherical aggregates. In spite of repeated attempts, no concordant analyses could be obtained indicating a homogeneous substance. This observation agrees with the experience recorded by Neuberg and Neimann [1905] of difficulty in obtaining a uniform product by the action of phenylhydrazine upon glycuronic acid.

Attempts to prepare a *p*-bromophenylhydrazone resulted in the production of brown resin. Similarly attempts to prepare a crystalline dinitrophenylhydrazone were unsuccessful.

Conclusion.

An attempt has been made to elucidate the nature of the cell wall constituents of the Laminariaceae known as fucoidin and alginic acid. The former has been shown to be the calcium salt of a sulphuric acid ester of a non-reducing polymerised uronic acid complex combined with a methylpentosan, since it yields on hydrolysis sulphate ions and the methylpentose fucose; the nature of the uronic acid has not been established.

Alginic acid has been shown to be a polymerised form of the hitherto undescribed mannuronic acid, and to contain no pentosan or methylpentosan groupings. These facts emphasise the distinctive metabolism of the brown

algae, which appears to be founded upon a mannose, rather than a glucose basis—as is shown by the known occurrence of mannitol and mannitan [Haas and Hill, 1929], and the apparent absence of glucose in any appreciable amounts.

Summary.

1. Evidence is furnished that the substance described by Kylin as fucoidin is an ethereal sulphate. The substance contains also some uronic acid complex the nature of which has not been determined.

2. Alginic acid occurs mainly as such in the free state in the cell wall and to a limited extent only in the form of a water-soluble calcium magnesium alkali metal salt.

3. Alginic acid from *Laminaria* yields on hydrolysis mannuronic acid whose quinine salt is here described for the first time (see Addendum).

No evidence could be obtained of the formation of sugars by the hydrolysis of alginic acid and this substance cannot therefore be regarded as a source of any of the sugars which have been obtained by the hydrolysis of *Laminaria* weed as described by Müther and Tollens [1904], Manske [1930] and others.

4. Alginic acid on oxidation with nitric acid yields mannosaccharic acid.

5. Fucose obtained by the hydrolysis of the complete weed of *Laminaria* is derivable in part from fucoidin and in part possibly from the cell wall residue remaining after exhaustive extraction with sodium carbonate, but none is produced from alginic acid.

Addendum (March 27th, 1931).

Since the above was written it was found that when the solution obtained by heating alginic acid with 2 % sulphuric acid was further heated for 8 hours with 5 % sulphuric acid the resulting liquid, on treatment with cinchonine sulphate, yielded the low-melting salt, M.P. 161° and $[\alpha]_D = 154°$. This shows definitely that the nature of the cinchonine salt obtained depends upon the strength of acid and the time employed in the hydrolysis, and that the cinchonine salt of low melting point is not obtained solely from the further hydrolysis of the residue remaining after initial treatment with 2 % sulphuric acid (as implied on p. 407), but may actually be obtained by further hydrolysis of the solution which previously yielded the salt of high melting point.

Attempts to prepare two different quinine salts from the two sources which yielded the two distinct cinchonine salts gave the following results: hydrolysis with 2 % acid yielded a quinine salt of M.P. 168° and $[\alpha]_D = -173{\cdot}5°$, while hydrolysis with 5 % acid yielded a quinine salt of M.P. 162–163° and $[\alpha]_D = -175{\cdot}3$. The difference between the physical constants in these two cases is hardly sufficient to justify the conclusion that the two salts are really distinct substances, but on the other hand the existence of two cinchonine salts requires the existence of two quinine salts.

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XLVIII. NOTE ON DIHYDROXYACETONE.

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The fact that dihydroxyacetone spontaneously polymerises [Levene and Walti, 1928] and also exists in two distinct crystalline modifications which are readily interconvertible [Abderhalden, 1911] makes it a matter of difficulty to prepare a consistently pure specimen. The commercial product as supplied by Meister, Lucius, Brüning and Co. under the name of "oxantin" which has been extensively used, gave variable results when tested physiologically, and we have shown [Reeves and Renbom, 1929] that on standing it becomes sticky and gives rise to appreciable quantities of lactic acid. The following typical treatment of the impure material gives a pure and stable compound.

Experimental.

Fifty grams of the commercial product were suspended in either 100 cc. of absolute alcohol, or better, pure acetone and shaken continuously for 24 hours at room temperature. After filtering and washing with the solvent, the precipitate was dried in a vacuum over $CaCl_2$ for 2 days. The filtrate was bright yellow in colour and strongly acid (p_H 5–5·5) and after removal of the solvent gave a clear yellow sticky mass which contained, in addition to lactic acid, traces of methylglyoxal as reported by Bernhauer and Wolf [1929]. 25·65 g. (51·3 % yield) of precipitate were thus obtained. Recrystallised from hot absolute alcohol it gave long colourless prismatic plates, soluble in H_2O, sparingly soluble in organic media and melting indefinitely at 82–83·5° to a colourless liquid. At the melting point a turbidity developed and re-solidification was difficult owing possibly to the occurrence of dissociation.

Analysis C 39·96 %, H 6·65 % (theoretical C 40 %, H 6·66 %); a cryoscopic determination of the molecular weight in aqueous solution gave $M = 130$ (theoretical for the bimolecular compound $M = 180$) similar to that obtained by Wohl [1898].

The crystals were further identified by preparing the phenylosazone, m.p. 132°. Hence the dihydroxyacetone obtained was the bimolecular α-modification. When distilled under reduced pressure (5–8 mm.) it gives a distillate which crystallises in colourless needles m.p. 68–71·5° and is unimolecular as determined cryoscopically in aqueous solution, $M = 86$ (theoretical $M = 90$)[1]. This

[1] Dr F. Dickens informs us that he has obtained a similar result from both the crude and the purified oxantin.

latter compound corresponds to that obtained by Fischer and Mildbrand [1924] and is evidently the β-modification.

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XLIX. THE EFFECT OF ARGININE UPON THE BODY WEIGHT OF MICE INJECTED WITH THYROXINE, AND BEARING THE TUMOUR M 63.

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In an earlier paper [Gilroy, 1930, 2], the hypothesis was advanced that thyroxine might modify the growth rate of neoplastic tissue, and in the case of a transplantable tumour of the mouse, M 63, this assumption was to a great extent confirmed. The suggestion that thyroxine exerted an inhibitory effect by inducing the catabolism of tissue protein also received support, for it was possible to cancel the effect characteristic of thyroxine if arginine was administered during the same experimental period, the results suggesting the maintenance of normal equilibrium in relation to supply and demand.

It remained to be decided whether the action of thyroxine was primarily upon the tumour, or whether the latter was only affected indirectly owing to some alteration of the metabolism of the host. The injection of 0·1 mg. of thyroxine daily for 12 days did not induce any toxic effects, but when the experiment was extended to 18 days 7 mice died out of a total of 47, from which it appeared that the threshold of toxicity had then been reached.

The records of weight showed an average gain which corresponded very closely with increase in tumour mass, and there was no evidence of any loss of body weight, although it seemed probable that some reduction in the latter would have occurred. The work of Abderhalden and Wertheimer [1928], and Romeis [1923], has shown that in spite of the relative insusceptibility of rats and mice towards thyroxine, a loss of weight is induced by the repeated administration of small amounts. It was evident that if total weight remained stationary, or diminished, while the tumour continued to grow, a reduction in body weight must have occurred. Observations were therefore confined to a determination of the number of mice bearing tumours of increasing size in which total weight either remained stationary or diminished.

Experimental.

The technique of implantation was described in an earlier paper [Gilroy, 1930, 1]. Treatment consisted in the daily injection of 0·1 cc. of a 0·1 % solution of thyroxine, or of 0·3 cc. of a 10 % solution of arginine; in one group

both substances were injected, but the arginine was injected in the morning and the thyroxine at night. The total duration of the experiment was 12 days, the first injections being given 10 days after implantation; in certain groups treatment was reversed halfway through the experimental period, and the data are therefore given in every case for the 16th and 22nd days after implantation.

Table I shows the number of mice in which weight was gained, remained stationary, or diminished, expressed as a percentage, the actual numbers being given in brackets below.

Table I.

No. of mice	Weight increased		Weight stationary		Weight decreased	
	Day 16	Day 22	Day 16	Day 22	Day 16	Day 22
	Arginine	Thyroxine	Arginine	Thyroxine	Arginine	Thyroxine
27	89·0	55·6	7·4	22·2	3·7	22·2
	(24)	(15)	(2)	(6)	(1)	(6)
	Thyroxine	Arginine	Thyroxine	Arginine	Thyroxine	Arginine
27	55·6	89·0	14·8	7·4	29·6	3·7
	(15)	(24)	(4)	(2)	(8)	(1)
	Arginine + thyroxine		Arginine + thyroxine		Arginine + thyroxine	
16	87·5	94·0	—	6·0	12·5	—
	(14)	(15)	(0)	(1)	(2)	(0)
	Arginine	Arginine	Arginine	Arginine	Arginine	Arginine
18	89·0	94·5	—	—	11·0	5·5
	(16)	(17)	(0)	(0)	(2)	(1)
	Thyroxine	Thyroxine	Thyroxine	Thyroxine	Thyroxine	Thyroxine
18	72·2	83·5	—	5·5	27·8	11·0
	(13)	(15)	(0)	(1)	(5)	(2)
	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated
20	95·0	75·0	—	10·0	5·0	15·0
	(19)	(15)	(0)	(2)	(1)	(3)

Owing to the small numbers involved no statistical significance can be attached to the results, but from the data presented certain inferences may be made. In a group of 27 mice injected with thyroxine for 6 days, 12 lost weight, while of 27 mice injected with arginine only 3 lost weight in the same number of days. Among 18 mice 8 lost weight when treatment with thyroxine was continued for 12 days and again only 3 out of 18 mice lost weight when injected with arginine for a similar period of time. When both arginine and thyroxine were injected for 12 days 3 out of 16 mice lost weight; this compares favourably with a loss of weight in 6 out of 20 in the group receiving no treatment at all.

Under ordinary circumstances, with normal stock, the injection of arginine does not appear to have any effect upon the body weight of mice, but it seems that if for any reason an undue strain is thrown upon the system of the animal, the tendency to lose weight can be compensated by the administration of this amino-acid. Thus the growth of a tumour absorbs certain nutritive substances at the expense of the tissues of the host; thyroxine increases the

demand for such materials by increasing general metabolism, and in both cases arginine seems capable of balancing the tissue loss. It may be inferred that arginine either exerts some protective action against the influence of thyroxine, or facilitates the immediate reconstruction of tissue protein.

The substances which are known to modify the effect of thyroxine upon metabolism fall into two groups; the first of these, exemplified by ergotamine [Abderhalden and Wertheimer, 1927], may be described as specific in their action; the second are non-specific and are represented by the carbohydrates. Arginine, being an inert substance, is clearly not to be included in the first category but it is possible that in the present instance arginine functioned as a precursor of sugar. That this might occur is shown by the studies of amino-acid metabolism in diabetes, from the results of which Dakin [1923], concluded that "arginine is the only amino-acid with more than five carbon atoms which can furnish glucose freely," the intermediate step being the formation of ornithine by the action of the enzyme arginase. It is therefore possible that under certain circumstances arginine may be glycogenic, and it is well known that a diet rich in sugar-forming materials can counteract the influence of thyroxine upon metabolism [Abderhalden and Wertheimer, 1926], while protein accentuates its effects.

Although arginine may serve as a source of sugar to the organism if need arises, this does not elucidate its action in accelerating the rate of tumour growth. It was shown by Cioffari and Piccaluga [1926], that a diet rich in carbohydrate did not have a stimulating effect, and in a previous series of experiments [Gilroy, 1930, 1], it was observed that amino-acids such as glycine and alanine were without any influence in increasing the rate of tumour growth, although these can be completely converted into glucose in the diabetic organism [Ringer and Lusk, 1910]. That arginine is an important constituent of tumours was shown by Kocher [1915], who found that it accounted for 12·4 % of the total protein nitrogen of neoplastic tissue. The specific importance of arginine in this connection was disputed by Drummond [1916], who pointed out that the diamino-acid content of any tissue was correlated with its nuclear content, and not with its rate of growth. It would seem, however, that the formation of nuclear material must involve the assimilation, or synthesis, of those substances which enter into its constitution, otherwise growth by cell division could not occur.

The alleged indispensability of arginine in general metabolism was questioned by Rose and Cox [1924], and Rose and Cook [1925], but more recently Scull and Rose [1930], have demonstrated in the case of rats the possibility of a synthesis of arginine in the body. It seems not unlikely that a synthetic mechanism may be provided by a reversal of the normal hydrolytic action of arginase, for it is known, from the experiments of Wasteneys and Borsook [1925], that proteolytic enzymes are also capable of synthesis. The high arginine content of tumours may perhaps be thus explained, since arginase has been shown by Edlbacher and Merz [1927], to be present in neoplastic

tissue in considerable amount. A tissue thus equipped would not be dependent upon preformed arginine for nuclear synthesis and would to this extent more readily attain optimum conditions for growth.

Summary.

126 mice were implanted with the carcinoma M 63, and 10 days later were divided into groups, 88 mice receiving injections of thyroxine in doses of 0·1 mg. daily for a period of 6 or 12 days. In certain groups arginine was injected coincidently with thyroxine; the remainder were either injected with arginine alone, or were given no treatment at all.

The mice were weighed individually three times during the experiment and the results obtained indicated that arginine prevented a loss of weight which thyroxine would otherwise have induced.

As a high carbohydrate diet is known to modify the influence of thyroxine upon metabolism it is suggested that, in this instance, arginine was utilised for the formation of sugar, the latter being derived from ornithine, but reasons are advanced for considering that the glycogenic function of arginine could not be responsible for increasing the rate of tumour growth.

I wish to record my indebtedness to the Medical Research Council for a grant which made this investigation possible, and to Professor Crew for scientific hospitality, and for his encouragement and advice.

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L. IS AVITAMINOSIS B_1 AN INTOXICATION BY METHYLGLYOXAL?

GLYOXALASE—CO-ENZYME RATIO IN EXPERIMENTAL BERIBERI.

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(Preliminary Communication.)

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In 1921 Findlay [1921] reported that "the glyoxalase content of the liver in pigeons with beriberi is less than that in control pigeons," and "the administration of vitamin B to a beriberic pigeon is followed by an increase in the glyoxalase content of the liver," but "vitamin B does not act as a co-enzyme of glyoxalase." Findlay drew no conclusion concerning the nature of the toxic substance supposed to come into existence during carbohydrate metabolism in the absence of the antineuritic vitamin. Since Findlay's discovery important investigations have been made both with respect to vitamin B and to the conditions of dismutation of methylglyoxal (and phenylglyoxal).

The complex nature of water-soluble vitamin B, *viz.* its resolution into the components B_1 (antineuritic and growth-promoting vitamin) and B_2 (P.P. factor, growth-promoting and pellagra-preventing vitamin) is now a well-known fact; it is possible that more components are contained in the vitamin B complex, but as yet this has not been definitely elucidated.

According to our present knowledge vitamin B_1 seems to be closely related to carbohydrate metabolism [Evans and Lepkovsky, 1929–30] and, through the latter, possibly also to protein metabolism. As to the importance of vitamin B_1 for fat metabolism it can only be said to be minimal; as yet the importance of vitamin B_2 in metabolism is unknown.

Several investigators have spoken in favour of the toxic pathogenesis of beriberi, none of them, however, stating the nature of the toxic substance with certainty; it was only supposed to come into existence during carbohydrate metabolism in the absence of vitamin B_1. The hypothesis most recently advanced [Peters, 1930], associated avitaminosis B_1 with an increase of the amount of lactic acid in the organism, especially of the brain. Peters believes that vitamin B_1 is concerned with the removal of lactic acid in the Meyerhof carbohydrate cycle.

As far as I know the literature does not comprise any work associating beriberi with an intoxication by methylglyoxal.

As demonstrated by Toenniessen and Fischer [1926], Ariyama [1928, 1, 2] and Vogt [1929] methylglyoxal is, also in animal glycolysis, an intermediary substance in carbohydrate catabolism, a dismutation of methylglyoxal into lactic acid eventually taking place.

Formerly methylglyoxal was considered a non-toxic substance [Dakin and Dudley, 1913, 4] but this view proved to be erroneous; it is a toxic substance as shown by Sjolleme and Seekles [1926], Fischler [1927], Kermack, Lambie and Slater [1927], and Herring and Hynd [1928].

Normally methylglyoxal cannot be demonstrated in the organism, a dismutation of the substance by means of glyoxalase (ketonealdehydemutase) taking place in the presence of the co-enzyme (co-mutase). Neuberg and Kobel [1928, 1929, 1, 2] noticed that well-washed lactobacillus or yeast, which was capable of converting hexosediphosphate to methylglyoxal but incapable of forming lactic acid from methylglyoxal, acquired the ability of producing lactic acid when boiled yeast juice was added to it. This was interpreted by them as indicating that co-enzyme was indispensable for the enzymic production of lactic acid from methylglyoxal. It was also demonstrated by Vogt [1929] in the case of liver (and other organic) enzyme systems that no dismutation of methylglyoxal will take place in the absence of the co-enzyme; under these conditions methylglyoxal is stabilised as such, and dismutation will only take place when co-enzyme is added to the enzyme system. Glyoxalase (a much better, because more logical, term would be ketonealdehydemutase as suggested by Neuberg [1913, 1, 2]) is widely distributed in nearly all tissues, more especially in the liver and muscles; and besides its rôle in the metabolism of sugar it also plays a part in the conversion of *d*-alanine to lactic acid. The presence of glyoxalase in the tissues is thus of importance in relation to the metabolism of carbohydrates and proteins. The wide distribution of glyoxalase in the plant and animal worlds seems to afford a strong indirect support for the methylglyoxal theory.

In my experiments mice and pigeons were used and three different methods employed. (1) The technique of Dakin and Dudley [1913, 1, 2, 3] and Neuberg [1913, 1, 2] which consists essentially in allowing a 20 % watery extract of the tissues to act on methylglyoxal (phenylglyoxal), and when the methylglyoxal (phenylglyoxal) is converted into lactic acid (mandelic acid), extracting the acid and estimating it by titration in terms of $N/10$ NaOH. (2) The technique of Vogt (1929) which consists in precipitation of methylglyoxal with 2 : 4-dinitrophenylhydrazine as bis-hydrazone. (3) The colorimetric method introduced by Ariyama [1928, 1] which is based upon the fact that glyoxals acquire, by the addition of cyanide, such a great increase of reducing intensity in alkaline solution that they cause the colour development of Benedict's arsenophosphotungstic reagent. None of the possible intermediate products of sugar metabolism, such as formic, acetic, glycollic, and glyceric aldehydes,

lactic and pyruvic acids or free hexose, interferes with the determination by developing the blue colour, with the exception of dihydroxyacetone, which gives about 20 % of the colour of methylglyoxal of the same concentration.

I thus succeeded in proving that methylglyoxal cannot be demonstrated in hexosephosphate + liver enzyme systems in starved animals any more than in normal animals, but that this substance can be demonstrated in hexosephosphate + liver extract from animals that do not get vitamin B_1, *i.e.* in animals otherwise getting a complete diet, comprising vitamins A + D (cod-liver oil), B_2 (autoclaved yeast), and E (wheat germ oil). I succeeded in demonstrating that the ingestion of vitamin B_1 (tikitiki-extract or Peter's concentrate) by the animals experimented upon apparently removed the difficulty attending the dismutation of methylglyoxal into lactic acid, and I also succeeded in demonstrating that in liver enzyme systems from animals suffering from avitaminosis B_1 methylglyoxal (and phenylglyoxal) is only converted into lactic acid (mandelic acid) to a small extent, whilst it was impossible in liver enzyme systems originating from normal (or starved) animals to demonstrate the presence of added methylglyoxal after the mixture had been allowed to stand for 24 hours. According to our present knowledge the enzyme is present in the liver tissue of animals suffering from avitaminosis B_1, whilst it seems to be the co-enzyme that is lacking, as a boiled aqueous extract of a normal animal liver, when added to the enzyme system originating from animals suffering from avitaminosis B_1, brings about the dismutation. This proof however is not conclusive when considered in relation to Vogt's [1929] investigations, because aqueous boiled extract of liver is also capable, although to a small extent, of bringing about a dismutation of methylglyoxal, the more so as the experiments with co-enzyme originating from yeast did not give completely satisfactory results (compare Findlay's results [1921]). As yet it cannot be decided with certainty whether it is the enzyme or, more probably, the co-enzyme that is lacking; perhaps there is a change of concentration in the enzyme/co-enzyme ratio of the organs of animals suffering from beriberi.

The fact that there is a possibility of accumulating methylglyoxal in animals suffering from avitaminosis B_1 is the more interesting as the symptoms of methylglyoxal intoxication in many respects bear a resemblance to those of experimental beriberi. Possibly the low blood-sugar level in the terminal stage of avitaminosis B_1 plays a role in regard to the sensitiveness of the animal to methylglyoxal; it is interesting in this connection to note that polyneuritic symptoms in the mouse resemble insulin symptoms in the same animal, especially interesting because methylglyoxal possibly plays a part in the production of the symptoms of insulin hypoglycaemia.

The whole of the experimental material forming the basis of this preliminary communication will soon be published.

SUMMARY.

The hypothesis is set up that the symptoms of avitaminosis B_1 are caused, wholly or partly, by an intoxication by methylglyoxal occurring on account of a failure of the dismutation of methylglyoxal, the tissue being deprived of the co-enzyme (not glyoxalase itself), or at least of a greater quantity of co-enzyme in comparison with glyoxalase.

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LI. THE INACTIVATION OF TRYPSIN-KINASE BY HEAT, AND THE EFFECT OF ADDED PROTEIN THEREON.

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In previous papers the writer has described experiments on the inactivation of trypsin [1930] and of enterokinase [1931] by heat. The first section of the present paper deals with the inactivation by heat of trypsin-kinase, the complex formed by union of the enzyme trypsin with its specific activator enterokinase. The examination of the heat-inactivation of trypsin-kinase involves in principle the three following operations:

(1) the formation of trypsin-kinase by mixing solutions of trypsin and enterokinase and leaving the mixture for 30 mins. in a thermostat at 30°;

(2) the transference of the trypsin-kinase, resulting after the above period of activation, to a thermostat at 50°, and the removal of samples at various time intervals;

(3) the measurement of the trypsin-kinase content of the various samples by the extent of the hydrolysis produced in a caseinogen solution under standard conditions.

The general experimental procedure involved in these operations may now be briefly described.

Experimental procedure.

For comparison with the work on the heat-inactivation of trypsin free from enterokinase, where the glycerol concentration was 24 %, it is necessary to arrange in the present investigation that the solution of trypsin-kinase undergoing inactivation should have the same glycerol concentration. This is arrived at in the following way.

5 g. of dried intestinal mucosa, prepared according to the method of Waldschmidt-Leitz [1924], were dispersed through 200 cc. of a glycerol-water mixture (containing 23·6 % of glycerol) by shaking and the dispersion was left for 24 hours in the ice-chest to allow of the activator passing into pseudo-solution. The dispersion was then centrifuged and filtered, and to 160 cc. of the filtrate were added 3 cc. of *N* acetic acid. The resulting precipitate of protein matter was centrifuged off, and the clear supernatant liquid neutralised

with N ammonia and kept in the ice-chest. This kinase solution now contained 23 % of glycerol. The experiments were then carried out as follows.

15 cc. of trypsin solution prepared as described in a previous paper of the writer [1930] (and containing 26·4 % of glycerol) were added to 10 cc. of the kinase solution described above. Formation of trypsin-kinase was allowed to occur by maintaining the mixed solutions for 30 mins. at 30°. At the end of this period 1 cc. of p_H-adjusting liquid (N acetic acid, N ammonia and distilled water) was added, thus producing in the final solution a glycerol content of 24 %. Of this final solution 3 cc. were measured out into each of a number of flasks already immersed in a thermostat at 50°. These flasks were withdrawn into crushed ice at various time intervals and the quantity of trypsin-kinase, *i.e.* the number of "units" therein, determined by the method of Linderstrøm-Lang and Steenberg [1929] at 30°, as described by the writer in a previous paper [1931].

The p_H measurements were carried out at room temperature by means of the glass electrode.

It was found that the course of the reaction, using this solution of trypsin-kinase, is in fair agreement with the unimolecular expression[1]:

$$k = \frac{2{\cdot}303}{t} \log_{10} \frac{a}{a-x}$$

where a = initial content of trypsin-kinase,

$a - x$ = content of trypsin-kinase after time t mins.

Table I, which is typical of the results in general, illustrates the degree of agreement with the unimolecular expression.

Table I.

Temperature 50°. p_H 8·77. Glycerol 24 %.

Time (mins.)	Trypsin-kinase content units per cc.	$k \times 10^3$
0	1·00	—
30	0·83	6·21
60	0·67	6·67
100	0·54	6·16
220	0·25	6·30

The effect of p_H upon the heat-inactivation.

The stability of all enzymes is influenced very markedly by the p_H of the medium, and Table II shows the effect of p_H upon the heat-inactivation of trypsin-kinase. In the second column the mean of the values of k for a particular p_H are set down opposite that p_H. The third column shows "smoothed values" of k obtained from the most probable curve resulting on plotting the experimental values of k against p_H. This curve is shown in Fig. 1.

[1] Throughout this paper k represents the velocity constant of a unimolecular reaction.

Table II.

Temperature 50°. Glycerol 24 %.

p_H	$k \times 10^3$ Experimentally found	$k \times 10^3$ "Smoothed" from curve
4·10	4·9	4·9
4·20	4·7	4·7
4·97	3·3	3·4
5·09	3·3	3·2
5·90	2·1	2·3
5·98	2·4	2·2
6·50	1·9	2·0
6·57	2·2	2·05
7·88	3·4	3·5
8·15	4·6	4·3
8·67	6·6	6·3
8·77	6·3	6·9
8·92	9·6	9·6

It is seen that the optimum stability for trypsin-kinase lies about p_H 6·5.

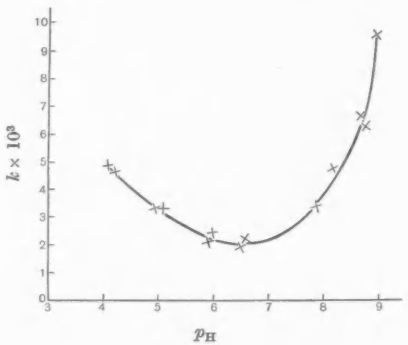

Fig. 1.

The critical increment for the heat-inactivation of trypsin-kinase.

Experiments were carried out to determine the critical increment for the heat-inactivation of trypsin-kinase. This was determined by measuring k at 50° and 60° and then substituting the mean values in the integrated form of the equation:

$$\frac{d \log_e k}{dT} = \frac{E}{RT^2}.$$

Measurements were made:

(1) in the region of optimum stability, p_H 6·5,

(2) in the acid region at p_H 4·1, p_H 5·1, p_H 5·9,

(3) in the alkaline region at p_H 8·8.

The results are summarised in Table III in which the figures in the last column represent the critical increments calculated by substituting the mean values of k at 50° and 60° into the integrated form of the equation.

Table III.

p_H	k (at 50°) $\times 10^3$	k (at 60°) $\times 10^2$	E (cals.)
8·8	6·34	4·77	43,000
6·5	1·90	1·55	44,600
5·9	2·14	1·35	39,000
5·1	3·27	2·80	45,000
4·1	4·95	2·90	38,000

The critical increment appears to vary with p_H in a fortuitous manner. It may be concluded that over the p_H range 4–8·8 it is of the order of 40,000 calories per "molar unit" of trypsin-kinase.

Comparison with the results obtained for trypsin[1] *free from enterokinase.*

It will be seen, on comparing these results with those obtained by the writer [1930] for the heat-inactivation of trypsin free from enterokinase, that the behaviour is similar in two important respects:

(1) in the position of the region of optimum stability, namely about p_H 6·5;

(2) in the order of magnitude of the critical increment, namely about 40,000 calories, being sensibly the same at the region of optimum stability and at points on the acid and alkaline sides in each case.

This similarity suggests that the active enzymic groupings may still be of the same nature in trypsin-kinase as they are in trypsin. Since however trypsin-kinase has more extensive proteolytic activity than trypsin alone it is to be inferred that the attachment of the kinase, necessarily at points other than those proteolytically active, serves to intensify the latter.

A comparison of the actual rates of heat-inactivation in both cases is of interest. This may possibly be best effected if, instead of comparing unimolecular constants, we compare the times of "half decomposition." This time interval is obtained from the unimolecular expression by writing

$$k = \frac{2{\cdot}303}{t_{\frac{1}{2}}} \log_{10} 2.$$

The comparison is made in Table IV. The values of k corresponding to the

Table IV.

Temperature 50°. Glycerol 24 %.

p_H	Time for "half decomposition" (mins.) Trypsin	Trypsin-kinase
5·0	231	210
6·0	346	277
7·0	346	300
8·0	247	193
8·5	198	128
9·0	144	74

[1] The word trypsin is used here in the sense employed by the Willstätter school up to 1930, *i.e.* the enzyme inactive towards true proteins but capable of hydrolysing certain protein degradation products. It has since been shown that trypsin itself may be resolved into a proteinase and a carboxy-polypeptidase. It is the latter fraction which is responsible for the hydrolysis of protein degradation products.

p_H-k curves for trypsin and trypsin-kinase respectively are calculated and then substituted into the equation for $t_{\frac{1}{2}}$ given above.

It is seen from Table IV that the rates of heat-inactivation of trypsin and of trypsin-kinase are fairly close together. On the whole it is to be concluded that trypsin is slightly more stable when free from its activator than when associated with it. This superior stability of trypsin over trypsin-kinase is most marked in the alkaline region.

The effect of the addition of protein upon the heat-inactivation of trypsin-kinase.

Proteins, peptones and amino-acids have been found to have a stabilising influence upon a number of enzymes. Luers and Lorinser [1922] found that egg-albumin and gelatin have a stabilising influence upon malt amylase, and Willstätter, Graser and Kuhn [1922] showed that leucylglycine and yeast gum protect saccharase to some extent from heat-inactivation. When the proteins and their degradation products are the substrates and products of reaction respectively, as with the proteolytic enzymes, the stabilising effect is most pronounced, for enzymes generally are found to be protected by their specific substrates and their products of reaction. Thus Biernacki [1891] found that albumins protected trypsin from heat-inactivation, and Bayliss and Starling [1904] have observed a similar effect with caseinogen peptones and egg-albumin. Northrop [1922] has stated that the products formed by the action of trypsin on proteins have a protective action upon the enzyme, but that gelatin itself has no such stabilising action.

In the following experiments, carried out by the writer, caseinogen was added to the solution of trypsin-kinase undergoing inactivation at two different p_H values:

(1) at p_H 8·5, *i.e.* in the region of optimum activity for the enzyme, where the products of reaction are most readily formed;

(2) at p_H 4·7, *i.e.* well outside the optimum region of activity for the enzyme.

The experimental procedure was as follows.

At p_H *8·5.* To 25 cc. of trypsin-kinase, prepared as described above, were added 2 cc. of a buffer, prepared by mixing equal volumes of N ammonia and N ammonium chloride, and 2 cc. of a 6 % caseinogen solution.

As a control the same solution was made up except that instead of 2 cc. of a 6 % caseinogen solution, 2 cc. of distilled water were used.

At p_H *4·7.* The same solution and control were made up as at p_H 8·5 except that 2 cc. of a buffer prepared by mixing equal volumes of 0·066 N sodium acetate and 0·066 N acetic acid were used instead of the alkaline buffer.

The solutions containing the added protein and also the control solutions were maintained at 50° for 90 mins. and the trypsin-kinase content was measured initially and at the end of this period of time. The results are shown in Tables V and VI.

Table V.

Temperature 50°. p_H 8·5.

	Time (mins.)	Trypsin-kinase units per cc. Run A	Run B	$k \times 10^3$
With added protein	0	1·06	1·00	Run A 3·3
	90	0·76	0·73	Run B 3·2
Without added protein	0	1·06	1·01	Run A 5·8
	90	0·63	0·61	Run B 5·6

From these results it follows that the mean decrease in k due to the added protein is 43 %.

Table VI.

Temperature 50°. p_H 4·7.

	Time (mins.)	Trypsin-kinase units per cc. Run A	Run B
With added protein	0	1·09	1·05
	90	0·85	0·81
Without added protein	0	1·09	1·05
	90	0·83	0·80

It is seen that at this p_H of 4·7 the difference between the degree of inactivation of the solution containing added protein and that of the control is within the experimental error.

It is to be concluded from these experiments that it is the products of hydrolysis which exert a stabilising influence upon the enzyme, the protein as such having little effect.

A further experiment was carried out in which the heat-inactivation was again determined at the alkaline p_H 8·5, but at 60° instead of at 50°. Since the critical increment for the tryptic hydrolysis of caseinogen is about 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930] while the critical increment for the heat-inactivation of the enzyme is seen to be of the order of 40,000 calories, a rise of 10° in temperature will result in a relatively smaller increase in the velocity of hydrolysis as compared with the increase in the velocity of inactivation. So that if, as we have inferred, the products of hydrolysis exert a stabilising influence upon the enzyme, this effect should be relatively less at 60° than it is at 50°. The results of the experiment are seen in Table VII.

Table VII.

Temperature 60°. p_H 8·5.

	Time (mins.)	Trypsin-kinase units per cc.	$k \times 10^2$
With added protein	0	1·02	3·6
	90	0·71	
Without added protein	0	1·02	4·0
	90	0·68	

The decrease in k owing to added protein is now 10 %, compared with the decrease of 43 % in the corresponding experiment at 50°. This result is therefore in agreement with the conclusion that it is the products of reaction rather than the protein itself which stabilise the enzyme to some extent against heat-inactivation.

In experiments carried out by the writer on the heat-inactivation of a sample of commercial "active trypsin" (*i.e.* trypsin-kinase) it was found that the constants calculated on the basis of the unimolecular expression decreased with increase in time. Northrop [1922] found the same behaviour in experiments with commercial trypsin. He put forward the explanation that the slowing up of the reaction is due to the enzyme acting upon the protein matter in the preparation used, when the resulting products of hydrolysis unite with the enzyme to form compounds which are more stable to heat than the free enzyme. This conclusion, arrived at by Northrop, is substantially confirmed by the results described in this paper.

SUMMARY.

1. A solution of trypsin-kinase has been prepared by the interaction of trypsin and enterokinase solutions for 30 mins. at 30°. The solution was used to study the heat-inactivation of trypsin-kinase. At the degree of purity employed it is found that the course of the reaction is in fair agreement with the unimolecular expression.

2. The effect of p_H upon the heat-inactivation has been investigated and it is found that the optimum stability is about p_H 6·5.

3. The critical increment of the heat-inactivation process has been determined at five p_H values, lying between p_H 4 and 9. It is found that the critical increment is of the order of 40,000 calories per "molar unit" of enzyme.

4. Experiments have been carried out upon the effect of adding caseinogen to the solution of trypsin-kinase undergoing inactivation. The caseinogen was added to solutions of the enzyme at p_H 8·5 (in the optimum region of activity for trypsin-kinase) and at p_H 4·7 (well outside the optimum region of activity) respectively. It was found that at p_H 8·5 the effect of the caseinogen, added initially, is to slow up the heat-inactivation of the enzyme, but that at p_H 4·7 there is no marked stabilisation. Thus it is concluded that it is the products of hydrolysis of the caseinogen which stabilise the enzyme. Further support for this conclusion was obtained by conducting the heat-inactivation of the enzyme, with caseinogen added initially, again at p_H 8·5 but at a temperature 10° higher. It was found that under these conditions the relative stabilising effect is reduced.

The above work was carried out under the direction of Professor W. C. M. Lewis, to whom the writer is indebted for help and advice. The writer also desires to express his thanks to Imperial Chemical Industries, Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool.

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LII. THE MOLECULAR WEIGHT OF VITAMIN A.

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(*Received February 9th, 1931.*)

Introduction.

Recent experiments on the problem of the relation between carotene and vitamin A bore evidence in favour of the view that in the animal body carotene is being transformed into vitamin A [Moore, 1929; Capper, 1930; Wolff *et al.* 1930]. This being so, carotene would be the vegetable source from which vitamin A is built up and should be considered as a "provitamin." An artificial conversion has not yet been obtained. Euler, Euler and Karrer [1929] have suggested that vitamin A might be a partly hydrogenated carotene, but no evidence has been found about the nature of their chemical relation.

It seemed important to us to determine the relative molecular size of the two substances. We therefore determined their diffusion constants in the same solvent. Were these constants found to be of almost the same order of magnitude, then the conclusion could be drawn that a simple chemical relation (*e.g.* a hydrogenation or an oxidation) existed.

However, the result was the reverse. The diffusion constants of carotene and vitamin A showed such a difference that their chemical relation cannot be a simple one. If the idea that one substance is converted into the other is right, the conversion cannot consist in simple hydrogenation, but must be a more radical transformation of the molecule. It must be stated here, that what we considered as "vitamin A," was determined by the reaction of Carr and Price (the blue colour developed by a solution of $SbCl_3$ in chloroform). We started from the point of view that up to now there has been evidence enough to consider this reaction as specific for the substance with vitamin properties when the absorption band is at 610 $\mu\mu$.

In this connection we should like once more to draw attention to the fact that the transformation *in vivo* was also detected by the increase of the blue colouring capacity of liver extracts after animals had been fed with carotene.

Materials.

Vitamin A preparations were obtained from the unsaponifiable part of sheep-liver extracts. After saponification, the light petroleum extract was concentrated, cooled and filtered. The solution now contained about 0·1 mg.

of solid *per* rat unit of vitamin A[1] and was by no means a pure vitamin solution as the actual active constituent was perhaps 2 % of the solid residue. Since indifferent substances do not appreciably influence each other's rates of diffusion provided that their solutions are not too concentrated, it seemed possible to work with these impure preparations. By evaporating the light petroleum *in vacuo* in a carbon dioxide atmosphere, adding xylene and evaporating half of the latter, a clear solution in xylene was obtained. Crystalline carotene was prepared from carrots and showed a M.P. of 174°. Considering the desirability of avoiding convection currents, when bringing the pure solvent above the very dilute carotene solution in order to effect diffusion, it was necessary to bring the solution to a higher specific gravity than that of the pure solvent. Therefore a certain amount of cholesterol was added. This amount was in Exps. 1 and 2 about 6 %, in Exps. 3 and 4 about 3 %.

The ratio of carotene to the total solid was in Exp. 2 1:630, in Exp. 3 1:60. The solutions of carotene in xylene proved extremely unstable, even when kept hermetically closed in a dark room. To get rid of this inconvenience in the second series of experiments a small quantity of pyrogallol was added to the solution which proved to be very effective in keeping the quantity of carotene unchanged for a considerable time if preserved in the dark.

Method of measuring the diffusion velocity.

In our experiments we used the diffusion apparatus of Cohen and Bruins [1923]. This consists of six glass discs, which may be rotated around a common axis, all being of exactly the same thickness. With the exception of the lowest and uppermost, all the discs are provided with three round perforations fitting exactly upon each other. When the discs are put together these perforations make three cylindrical spaces. The actual experiment is carried out by filling the lowest section of these cylinders with the solution to be investigated and the three higher compartments with the pure solvent. By suitably and very carefully rotating the discs, the upper three compartments are now brought above the lower one. After the diffusion has taken place for a certain time, the four compartments are again separated by rotating two alternate discs (Nos. 2 and 4). In this way the column of liquid is divided into four equal parts. Every one of these parts is analysed separately. From the ratio of the concentrations of the dissolved substance in the four layers the diffusion constant may be calculated. For a detailed description of the apparatus and of the calculation the original literature should be consulted.

A few simplifications of the original procedure were introduced[2]. As our analytical methods did not permit a very high exactitude, part of the precautions usually exercised in diffusion experiments were not considered im-

[1] We have to thank N. V. Organon, Oss, for having supplied us with this preparation.

[2] The diffusion apparatus was placed in the cellar of the van 't Hoff Laboratory, Utrecht (Director Prof. Dr E. Cohen).

perative in this case. In the first series of experiments, with both substances, we did not work with a thermostat, but wrapped the diffusiometer in cotton wool and took care that no sudden changes of temperature should occur. During the second series the apparatus was placed in the air-thermostat [Cohen and Bruins, 1923, p. 371], but the water in the thermostat did not circulate and therefore no auxiliary thermostat was needed. The rotation of the discs was not performed mechanically but very carefully by hand. Before starting the diffusion process, the apparatus was kept for 3 hours in the thermostat.

In all our experiments we used pure *m*-xylene (B.P. 139–139·5°) as a solvent. The discs were lubricated with glycerol in the first series. As this took up moisture from the air very easily, the edges were greased with soft soap. In the second series the discs were lubricated by a hydrogel of glycerol-amylum.

Calculation.

Each diffusion experiment gave us three concentration values for each cylindrical section, the average of which was used for the calculation of the diffusion constant. To this end the tables of Kawalki [1894] were used.

Intermediate values, not recorded in these tables, were found by linear interpolation. In this way we obtained from each experiment four values of x in the formula:

$$x = \frac{h'^2}{D.t}$$

where h' is the half of the height of one section, D is the diffusion constant, t is the time of diffusion. From these four values the average was calculated for each diffusion-experiment, their relative "weights" being taken into account [for details see Cohen and Bruins, 1923].

The time of diffusion in all experiments was about the same, hence the following values for the "weight" p of the various sections were used, the relative variations in the concentrations of the four sections being between $x = 0{\cdot}1296$ and $x = 0{\cdot}1936$, *i.e.* the part of the tables used in these calculations.

Section 1	$p = 529$
2	$p = 312$
3	$p = 178$
4	$p = 660$
	$p = 1679$

$$x_{av} = \frac{\Sigma px}{\Sigma p}.$$

The diffusion constant is found from the formula:

$$D_{av} = \frac{h'^2}{x_{av}.t}$$

For our diffusiometer $h'^2 = 0{\cdot}2376$ [Cohen and Bruins, 1924], D is expressed in cm.^{2} per 24 hours.

For the calculation of the diffusion constant at 20° we used a temperature

coefficient of 1·4 % per ° C. This is the temperature coefficient of the viscosity of *m*-xylene calculated from the data given by Thorpe and Rodger (Landolt-Börnstein tables).

Method of analysis.

In these diffusion experiments only the relative concentrations in single sections are to be determined, and it is not required to know the absolute concentration of the substance investigated. This is very important for the reason that only in view of this fact may we use without any objection the reaction of Carr and Price [1926] for our vitamin A determinations. However doubtful the absolute value of this reaction is, the units by which we express the concentration value of the cylindrical sections may be quite arbitrary.

A. *Vitamin A determination.*

The reaction was carried out in the usual way (0·2 cc. of the solution, to which two drops of acetic anhydride were added to prevent precipitation, were mixed with 2 cc. of a saturated solution of $SbCl_3$ in chloroform [Bertram, 1929], and the blue colour measured in the Lovibond colorimeter.

The colour expressed in Lovibond units not being a linear function of the dilution, a standard curve of this function was made.

The solutions to be investigated were always brought to such dilutions that the reaction of Carr and Price gave a blue colour corresponding to about 5 units. Two other dilutions were made, if possible of about 4 and 6 units. From these three determinations the average value was obtained. The values recorded in the Tables are these averages calculated in rat units of Rosenheim per cc. solution (5 Lovibond units are equal to 5 rat units). As already stated, the absolute value of the units is of no importance. The error in our series of experiments amounts to about 5–10 %. Errors of this range of magnitude seemed rather disturbing for the reliability of the diffusion constants to be calculated; therefore the average of three concentration measurements was used in these calculations.

B. *Carotene determinations.*

The determinations of carotene were carried out colorimetrically with the stuphometer of Zeiss, by means of a standard curve established in a preliminary series with solutions of known concentration. Carotene having an absorption band at 490–475 $\mu\mu$ the filter S_{47} was used in these determinations. Solutions of carotene containing 0·001–0·01 mg. per cc. can be determined very exactly with the photometer. The probable error in these estimations does not exceed 3 %. In the following tables the concentrations are recorded in 0·001 mg. per cc. These figures also represent the average of three determinations.

Results of the experiments.

Vitamin A; first series.

I.

Diffusion time: 3·030 days; temp. 16·3°–15·0°; average 15·7°.

	A	B	C	Average	0/000	x	$p.x$
1	73	—	72	72	1109	0·1477	78·13
2	127	—	123	125	1926	0·1397	43·59
3	188	—	193	191	2943	0·1226	21·82
4	269	—	253	261	4021	0·1526	100·72
				649	9999		244·26

$x_{av} = \frac{244·26}{1679} = 0·1455$

D = 0·540 cm.²/24 hours; D_{20} = 0·572 cm.²/24 hours.

II.

Diffusion time: 3·018 days; temp. 16·1°–18·2°; average 17·2°.

	A	B	C	Average	0/000	x	$p.x$
1	—	47	48	47·5	1237	0·1343	71·05
2	—	77	62	69·5	1811	0·1613	50·33
3	—	111	102	106·5	2773	0·0835	14·86
4	—	157	164	160·5	4180	0·1696	111·94
				384·0	10001		248·18

$x_{av} = \frac{248·18}{1679} = 0·1478$

D = 0·531 cm.²/24 hours; D_{20} = 0·552 cm.²/24 hours.

III.

Diffusion time: 3·015 days; temp. 18·9°–19·5°; average 19·2°.

	A	B	C	Average	0/000	x	$p.x$
1	58	58	—	58	1090	0·1495	79·09
2	107	107	—	107	2012	0·1222	38·13
3	158	157	—	158	2971	0·1242	22·11
4	208	210	—	209	3929	0·1452	95·83
				532	10002		235·16

$x_{av} = \frac{235·16}{1679} = 0·1401$

D = 0·564 cm.²/24 hours; D_{20} = 0·570 cm.²/24 hours.

Vitamin A; second series.

I.

Diffusion time: 2·950 days; temp. 19·3°–19·7°; average 19·5°.

	A	B	C	Average	0/000	x	$p.x$
1	33	32	34	33·0	1235	0·1347	71·26
2	52·5	52·5	52·5	52·5	1965	0·1310	40·87
3	77·5	77·5	79	78·3	2932	0·1151	20·49
4	105	105	100	103·3	3868	0·1398	92·27
				267·1	10000		224·89

$x_{av} = \frac{224·89}{1679} = 0·1339$

D = 0·602 cm.²/24 hours; D_{20} = 0·606 cm.²/24 hours.

II.

Diffusion time: 2·840 days; temp. 16·8°–17·3°; average 17·0°.

	A	B	C	Average	0/000	x	$p.x$
1	25	27	29	27·0	1049	0·1540	81·47
2	46·5	48	51·5	48·5	1885	0·1465	45·71
3	73	80	73	75·3	2927	0·1142	20·33
4	110	107	102·5	106·5	4139	0·1653	109·10
				257·3	10000		256·61

$x_{av} = \frac{256·61}{1679} = 0·1528$

D = 0·547 cm.²/24 hours; D_{20} = 0·569 cm.²/24 hours.

III.

Diffusion time: 3·042 days; temp. 19·5°–19·9°; average 19·7°.

	A	B	C	Average	0/000	x	$p.x$
1	48	46	46	46·7	1253	0·1327	70·20
2	73	73	75	73·7	1978	0·1285	40·09
3	108	107	112	109·0	2926	0·1141	20·31
4	142·5	148	139	143·2	3843	0·1376	90·82
				372·6	10000		221·42

$x_{av} = \frac{221{\cdot}42}{1679} = 0{\cdot}1319$

D = 0·592 cm.²/24 hours; D_{20} = 0·594 cm.²/24 hours.

Carotene; first series.

I.

Diffusion time: 2·930 days; temp. 19·3°–16·8° (mostly 17·8°–16·8°) average 17·3°.

	A	B	C	Average	0/000	x
1	39·5	40·5	—	40	851	0·1802
2	83	79·5	—	81	1724	0·1798
3	162	160·5	—	161	3426	—
4	193·5	182·5	—	188	4000	0·1518
				470	10001	

Section 1 + 2 = 2575; section 3 + 4 = 7426; x = 0·1803; D = 0·450 cm.²/24 hours; D_{20} = 0·467 cm.²/24 hours.

II.

Diffusion time: 2·920 days; temp. 18·2°–17·6°; average 17·9°.

	A	B	C	Average	0/000	x	$p.x$
1	—	8·6	9·0	8·8	962	0·1647	87·13
2	—	14·2	13·9	14·05	1536	0·2228	69·51
3	—	28·8	30·0	29·4	3215	0·2288	40·73
4	—	38·4	40·0	39·2	4287	0·1807	119·26
				91·45	10000		316·63

$x_{av} = \frac{316{\cdot}63}{1679} = 0{\cdot}1886$

D = 0·431 cm.²/24 hours; D_{20} = 0·444 cm.²/24 hours.

Carotene; second series.

I.

Diffusion time: 2·816 days; temp. 17·2°–16·5°; average 16·9°.

	A	B	C	Average	0/000	x	$p.x$
1	36	36	35·5	36	754	0·1935	102·36
2	80	78	77·5	78·5	1644	0·1973	61·56
3	148·5	147·5	148·5	148	3100	0·1614	28·73
4	215·5	214	215	215	4502	0·2046	135·04
				477·5	10000		327·69

$x_{av} = \frac{327{\cdot}69}{1679} = 0{\cdot}1952$

D = 0·432 cm.²/24 hours; D_{20} = 0·450 cm.²/24 hours.

II.

	A	B	C	Average	0/000	x	$p.x$
1	28	27	27·5	27·5	872	0·1773	93·79
2	53·5	53·5	53·5	53·5	1698	0·1854	57·84
3	100	97·5	98·6	99·0	3143	0·1806	32·15
4	135·5	136·5	132	135·0	4287	0·1807	119·26
				315·0	10000		303·04

$x_{av} = \frac{303{\cdot}04}{1679} = 0{\cdot}1805$

D = 0·439 cm.²/24 hours; D_{20} = 0·457 cm.²/24 hours.

Note. In Exp. 1 of the first series referring to carotene the concentration of section 3 was found very much too high and correspondingly the concentration of section 4 too low. Probably convection took place here.

In calculating the diffusion constant the sections 1 and 2 and 3 and 4 were added together and the value of x calculated by interpolating for the sum of these concentrations in the tables of Kawalki.

Discussion of the results.

The following table contains the summary of results referring to the diffusion constants:

Vitamin A.

	First series	Second series
I	$D_{20}=0{\cdot}572$	$D_{20}=0{\cdot}606$
II	$D_{20}=0{\cdot}552$	$D_{20}=0{\cdot}569$
III	$D_{20}=0{\cdot}570$	$D_{20}=0{\cdot}594$
Average	0·565	0·590

Average $D_{20}=0{\cdot}578$ cm.²/24 hours.

Carotene.

	First series	Second series
I	$D_{20}=0{\cdot}467$	$D_{20}=0{\cdot}450$
II	$D_{20}=0{\cdot}444$	$D_{20}=0{\cdot}457$
Average	0·456	0·454

Average $D_{20}=0{\cdot}455$ cm.²/24 hours.

The diffusion constant of vitamin A is found to be considerably higher than that of carotene, the difference exceeding appreciably the probable error of the experiments.

The results of parallel estimations of the vitamin experiments do not agree amongst each other as accurately as do other diffusion experiments done with the same apparatus. Probably the method of analysis must be considered as the principal source of inexactitude; as already stated the probable error of one determination amounts to 10 %. The accuracy of the observations was however enhanced by their number, considering that every value of the diffusion constant as recorded above is based on the average of 36 analyses. The accuracy of the analytical method for carotene exceeds that for vitamin A; this is perhaps the reason why the values found for carotene are more consistent than those for vitamin A.

Other sources of errors are the following.

1. Fluctuations of temperature; these were rather great for diffusion experiments. Xylene is not an advantageous solvent for these determinations. It has a small specific heat, a high dilatation coefficient and a low viscosity (drawbacks which however it shares with most other organic solvents). The occurrence of convection currents is very much favoured by these properties.

2. Evaporation of the xylene while filling and emptying the diffusion cylinders.

3. In the first series some glycerol was found at the bottom of the diffusion cylinder, after the experiments were finished.

4. Oxidation of the substance investigated.

Though the influence of these four sources of error upon the ultimate result is perhaps inferior to that involved by inadequacy of the analyses they doubtless vitiate the results to some extent. We estimate the probable error in the final figures above recorded (provided that there are no intrinsic causes due to systematic errors), for vitamin A 4 %, for carotene 2 %.

The molecular weight of vitamin A.

As to the molecular weight of vitamin A we may state the following. For the relation between the diffusion constant and the molecular weight, an empirical formula has been found:

$$D\sqrt{M} = c,$$

in which D is the diffusion constant, M the molecular weight and c a constant, dependent on the nature of the solvent and the temperature. This formula was experimentally tested by Öholm [1909] and found to be fairly satisfactory.

By comparing the diffusion constants found above with the molecular weights we obtain the following result:

$$\frac{\sqrt{M_{car}}}{\sqrt{M_{vit}}} = \frac{D_{vit.}}{D_{car}} = \frac{0{\cdot}578}{0{\cdot}455} = 1{\cdot}27.$$

If we now substitute in this formula the known value for the molecular weight of carotene we obtain:

$$M_{vit} = \frac{M_{car}}{1{\cdot}27^2} = \frac{536}{1{\cdot}61} = 333.$$

According to these calculations the molecular weight of vitamin A should be equal to 333, a figure which may be accepted only with reserve considering the uncertainty about the determination of the diffusion constant of vitamin A, and the error in M being twice as much as the error in D.

In addition we emphasise that the formula used above is not founded on the exact kinetic theory of diffusion; according to this the diffusion constant is connected more closely with the size, *i.e.* the diameter of the particles, than with their weight.

SUMMARY.

The diffusion constants of carotene and vitamin A in xylene were determined and found to be 0·455 cm.²/24 hours, and 0·578 cm.²/24 hours, respectively at 20°. The difference found exceeds considerably the probable errors of measurement. The ratio of the molecular weights of the two substances has been calculated and from this a molecular weight of about 330 has been deduced for vitamin A. The value obtained causes the assumption of a simple chemical relation between vitamin A and carotene to appear improbable.

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LIII. INTERMEDIARY CARBOHYDRATE METABOLISM.

THE EFFECT OF SODIUM IODOACETATE ON GLYOXALASE.

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(*Received February 11th, 1931.*)

Introduction.

The keystone of a theory of intermediary carbohydrate metabolism, put forward by Dakin and Dudley [1913, 3; 1914] is the ketonic aldehyde, methylglyoxal. The postulation of this substance as the catabolic precursor and anabolic successor of lactic acid ($CH_3.CO.CHO \rightleftharpoons CH_3.CHOH.COOH$) correlates in an intelligible scheme many of the known metabolic changes of carbohydrates.

The discovery of the enzyme glyoxalase [Dakin and Dudley, 1913, 1], which, detectable in all mammalian tissues with the exception of the pancreas, rapidly converts methylglyoxal into lactic acid, provided substantial support for the theory, and a large body of evidence, subsequently collected, has upheld the opinion that methylglyoxal is, in fact, an intermediary compound in normal glycolysis [see *e.g.* Toenniessen and Fischer, 1926; Ariyama, 1928, 2].

Now Lundsgaard recently [1930, 1] independently rediscovered the fact, first noted by Pohl [1887], that the muscles of animals, poisoned with iodoacetic (or bromoacetic) acid, form no lactic acid under conditions in which normal muscle produces considerable amounts of this substance. Here then is a simple chemical substance which prevents the normal breakdown of carbohydrate, presumably by interfering with one or more of the catabolic reactions involved, and possibly, therefore, with that brought about by glyoxalase. The experimental examination of this possibility, which forms the basis of this paper, proves that sodium iodoacetate is in fact a powerful inhibitor of the action of glyoxalase.

Experimental.

In all the experiments here mentioned the breast-muscle of chickens was used as the source of the enzyme, since this tissue yields highly active extracts.

The technique of determining glyoxalase activity was that of Dakin and Dudley [1913, 4] in which the muscle extract is incubated with phenylglyoxal

in the presence of an excess of freshly precipitated calcium carbonate. *l*-Mandelic acid is produced, and a determination of its optical rotation gives a measure of the enzymic activity.

Glyoxalase is very sensitive to acids [Dakin and Dudley, 1913, 2] and it is for this reason that calcium carbonate is used to neutralise the acid formed during the reaction. It was obviously not permissible, therefore, in experiments on the effect of iodoacetic acid on the enzyme, to add the acid itself, and solutions of the neutral sodium salt were employed.

Numerous experiments concerning the effect of this salt on the action of glyoxalase *in vitro* were performed; in the interests of economy of space only one of these is described, which displays all the points of interest revealed in earlier experiments from which the best conditions for demonstrating the inhibitory effect of sodium iodoacetate were evolved.

Effect of sodium iodoacetate on the action of glyoxalase in vitro.

The minced breast-muscle of two chickens, which had been killed with chloroform, was digested with five times its weight of distilled water with frequent stirring at room-temperature for 2 hours. The extract was then filtered through muslin.

In the meantime a solution containing 3 mg. iodoacetic acid, neutralised with its equivalent of sodium hydroxide, per cc., a solution containing 20 mg. phenylglyoxal per cc., and an aqueous suspension of freshly precipitated calcium carbonate had been prepared.

All these liquids were first warmed to 37° and then in each of 8 flasks were placed 10 cc. of calcium carbonate suspension, 50 cc. of muscle extract and 2 drops of toluene. Each of the flasks 1–5 then received 10 cc. of sodium iodoacetate solution, and each of the flasks 6–8 (controls) 10 cc. of water. The flasks were kept at 37° and 10 cc. of phenylglyoxal solution were added to the series as follows: immediately to flasks 1 and 6; after 1 hr. to flask 2; after 2 hrs. to flasks 3 and 7; after 3 hrs. to flask 4; after 4 hrs. to flasks 5 and 8.

Each flask was withdrawn from the hot-room 20 hrs. after it received its phenylglyoxal, and the contents were worked up as follows.

25 g. ammonium sulphate were added and the flask was heated in a boiling water-bath for 5 mins. To the cooled contents were added cautiously 8 cc. of syrupy phosphoric acid. After standing for 30 mins. the liquid was filtered through a Büchner funnel; the precipitate was not washed. The filtrate was extracted 4 times with 10 cc. quantities of ether, and the combined extract was washed with 5 cc. water. The ether was then removed *in vacuo* and the residue dissolved in 7 cc. water. After filtration through paper in which a very small amount of charcoal had been placed the rotation of the Hg_{5461} line was read in a 1 dm. tube.

The experimental result is given in Fig. 1.

The points on curve *A* represent the rotations of the *l*-mandelic acid formed

in flasks 6, 7 and 8 and indicate that no appreciable loss of enzymic activity occurs when a glyoxalase solution is incubated in the presence of calcium carbonate at 37° for 4 hrs. before adding the substrate. The points on curve *B* represent the rotations of the *l*-mandelic acid formed in flasks 1–5 and the curve shows the rate of inactivation of glyoxalase by sodium iodoacetate under the prescribed experimental conditions.

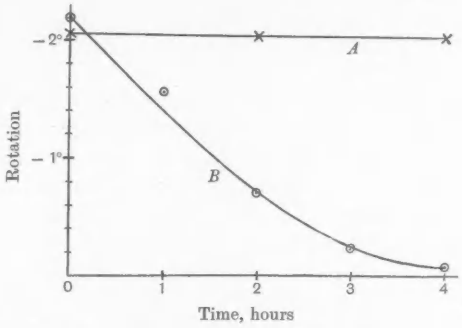

Fig. 1. *A* is the control glyoxalase curve. *B* shows the inactivation of glyoxalase by sodium iodoacetate.

To ensure comparable results in experiments of this kind the technique must be rigidly constant.

It is advisable to use flasks of equal capacity and shape. They should be large enough to allow the reaction mixture to lie in a shallow layer so that the calcium carbonate, which sinks to the bottom, may act reasonably efficiently as neutraliser of the acid formed. In these experiments 350 cc. Erlenmeyer flasks were employed.

The amount of toluene added as an antiseptic should be small and accurately measured since this substance itself has a definite inhibitory action on glyoxalase [Dakin and Dudley, 1913, 4].

The ether used for extracting the mandelic acid in experiments where iodoacetate has been added should be reasonably pure. Ether containing much peroxide liberates free iodine during the extraction. If this occurs a few drops of thiosulphate solution should be added to the water used for washing the combined ether extract.

Effect of sodium iodoacetate on glyoxalase in vivo.

4 cc. of a solution of sodium iodoacetate, equivalent to 26 mg. iodoacetic acid, were injected under the skin of the back into a chicken weighing 385 g. The first symptoms were noticed after 20 mins., the bird becoming lethargic. Its weakness steadily increased, until after $2\frac{3}{4}$ hrs. it died apparently from circulatory failure. A normal control bird of about the same weight was killed with chloroform and the breast-muscle of each was minced and digested for $2\frac{1}{2}$ hrs. at room temperature with five times its weight of distilled water. 50 cc. of each extract, filtered through muslin, were incubated at 37° for 16 hrs. with 10 cc. of calcium carbonate suspension and 10 cc. of a solution containing 0·2 g. phenylglyoxal, 2 drops of toluene being added to each flask as antiseptic. The mandelic acid formed was extracted in the manner already

described and the rotations were observed under the same conditions as in the previous experiment.

A second experiment in which a bird weighing 610 g. received 40 mg. iodoacetic acid as sodium salt in 4 cc. water was performed. The poisoned bird died $3\frac{1}{4}$ hrs. after the injection, and a control bird was killed for comparison at the same time. The rotations of the *l*-mandelic acid formed in the two experiments were as follows:

Normal chickens	Poisoned chickens
−2·06°	−1·24°
−2·02°	−1·19°

Although in many instances it is not justifiable to take tissues of one normal animal as controls against which to compare corresponding tissues of an experimental animal, it is the writer's experience that, with a rigid adherence to technique, the glyoxalase activity of a given tissue in a series of normal animals of the same kind is remarkably constant. The results quoted above are therefore considered to be significant, and the effect of poisoning with iodoacetic acid appears to have reduced the glyoxalase activity of the breast-muscle of these chickens nearly to one-half that of the same muscle of normal birds at a time when death supervenes.

Discussion.

The experiments described in this communication demonstrate that sodium iodoacetate is a powerful inhibitor of the tissue enzyme which converts methylglyoxal into lactic acid.

The significance of this observation is considerably enhanced by Lundsgaard's investigations which have shown that sodium iodoacetate is not a general enzymic poison; it disturbs neither glycogenolysis nor the normal breakdown of phosphagen in a muscle in which it has completely suppressed the formation of lactic acid [Lundsgaard, 1930, 2]. In particular is it noteworthy that it does not interfere with the actions of the carbohydrate-splitting enzymes invertase and ptyalin, and, whilst it prevents the alcoholic fermentation of yeast, the oxidative metabolism is unaffected by it [Lundsgaard, 1930, 2, 3]. In the light of these interesting experiments on the scope of its action Lundsgaard forms the opinion that sodium iodoacetate inhibits specifically the process of glycolysis, interfering, therefore, at some stage of the degradation of glucose to lactic acid. The work here presented indicates a specific inhibition of the reaction which has been postulated as that immediately concerned with the production of lactic acid in tissues, thus supporting at the same time Lundsgaard's conclusion and the view that methylglyoxal is indeed the immediate precursor of lactic acid in the normal glycolytic chain of reactions.

Another inhibitor of the action of glyoxalase has been known for some time. In the course of their early work on glyoxalase Dakin and Dudley [1913, 4] observed that pancreatic extracts inhibited the action of the enzyme,

and they named the factor "antiglyoxalase." It is very interesting to note that both antiglyoxalase and sodium iodoacetate exert their effect on the enzyme in the same manner; neither inhibitor acts instantaneously, the degree of inhibition increasing with the length of time of contact between inhibitor and enzyme. The curve shown in Fig. 1 is of precisely the same type as is obtained in similar experiments with antiglyoxalase, and could be closely imitated by using an appropriate pancreatic extract.

Some time after Dakin and Dudley's description of antiglyoxalase Winfield and Hopkins [1915] announced that pancreatic extract was capable of inhibiting the formation of lactic acid in muscle.

The assumption of identity between antiglyoxalase and Winfield and Hopkins's factor was rendered difficult at that time mainly by their statement that the latter was heat-stable (and could therefore be neither trypsin, amylopsin nor lipase) whilst Dakin and Dudley had found that antiglyoxalase was readily destroyed by heat. Further differences appeared when Foster [1925] claimed to have separated them by means of 70 % alcohol in which the lactic acid-inhibiting factor was said to be soluble whilst antiglyoxalase was not. On this point, however, her evidence does not carry full conviction, for in several of her experiments the alcoholic extracts displayed antiglyoxalase action. It is true that this was slight in comparison with the inhibition of lactic acid formation in muscle caused by these preparations, but since she allowed her extracts to act on glyoxalase solutions for only 1 hr. before adding the substrate, it is clear, remembering that antiglyoxalase develops its inhibitory effect in a manner closely similar to that shown for iodoacetate (Fig. 1), that only slight inhibition would be expected, and that this would bear no simple quantitative relationship to the results which she obtained on the inhibition of lactic acid production in muscle.

Some confirmation of the inhibitory factor's heat-stability, as reported by Winfield and Hopkins, was perhaps provided by the partial survival of activity in her preparations after autoclaving for 20 mins. at 120°, although it should be noted that they were tested only with respect to antiglyoxalase.

When phenylglyoxal is incubated in muscle extracts which have been inactivated with respect to glyoxalase, or in pancreatic extracts which contain none of this enzyme, a yellow colour, or yellow precipitate, appears. Foster considered that this phenomenon most probably indicated that pancreatic extract acted on the substrate rather than on the enzyme, and doubted the existence of "antiglyoxalase" in the sense postulated by Dakin and Dudley. This conclusion has been negatived by Ariyama [1928, 1], and recently Giršavičius [1930] has stated that the production of the yellow colour is entirely independent of the antiglyoxalase activity of the pancreas. Being apparently unaware of Dakin and Dudley's observation [1913, 1] that phenylglyoxal readily condenses with arginine, ornithine, histidine and lysine, yielding sparingly soluble yellow compounds, he rediscovered the clue to the proper explanation of the phenomenon, which is undoubtedly caused by

condensation of phenylglyoxal with basic amino-acids either in, or arising from, the protein of the tissue extracts.

Further investigations into the nature of the substance in pancreatic extracts which inhibits the formation of lactic acid by muscle have led McCullagh [1928] and Case and McCullagh [1928] to the conclusion that it is merely amylase, which acts by virtue of its power of preventing the formation of hexosephosphates, an explanation which has been challenged by Harrison and Mellanby [1930].

This conclusion is incompatible with Winfield and Hopkins's original observation on the heat-stability of the factor and with Foster's statement concerning its solubility in 70 % alcohol, for Harrison and Mellanby found that such solutions were practically devoid of amylolytic action.

As the problem stands at present, therefore, assuming the accuracy of the experimental observations briefly reviewed, it appears possible that there may be more than one mechanism whereby pancreatic extracts prevent the formation of lactic acid in muscle.

The significance and importance of antiglyoxalase in this connection is apparent from the work of Toenniessen and Fischer [1926; see also Ariyama, 1928, 2]. They have presented strong evidence that in a mixture of minced muscle and pancreas (or in mixed extracts of these organs) to which sodium fructosediphosphate had been added, methylglyoxal accumulated in detectable amounts. Similar experiments, using sodium iodoacetate as inhibitor, would be of considerable interest. According to Case and McCullagh's results, pancreatic amylase (or some associated substance) may interfere at an earlier stage of the degradation of carbohydrate; and the fact that a simple salt, sodium iodoacetate, inhibits lactic acid formation in muscle and the glyoxalase activity of muscle extracts, suggests the idea that there may occur in the pancreas a relatively simple compound, possibly heat-stable and soluble in 70 % alcohol, which also exerts an inhibitory power on glyoxalase, or on some other enzyme concerned in the process of normal glycolysis.

If this proved to be so, the conflict of evidence on the subject which has arisen in connection with this problem would be resolved.

In any event, however, there is good ground for the belief that the antiglyoxalase of pancreatic extracts does inhibit the formation of lactic acid in muscle by virtue of its inhibitory action on glyoxalase; and the fact that sodium iodoacetate, which suppresses the formation of lactic acid in muscle, also inhibits the action of glyoxalase, provides significant support to that belief.

SUMMARY.

1. Sodium iodoacetate, which Lundsgaard has shown to suppress lactic acid formation in muscle, is a powerful inhibitor of the enzyme glyoxalase which converts methylglyoxal into lactic acid. The available evidence supports the view that sodium iodoacetate owes its power of interfering with normal glycolysis to this fact.

2. The probability that methylglyoxal is the immediate precursor of lactic acid in the glycolytic chain of reactions is strengthened by this observation.

3. The inhibitory actions of sodium iodoacetate and pancreatic anti-glyoxalase on glyoxalase are similar in character: the significance of this finding is discussed in connection with the conflicting reports on the nature of the factor in pancreatic extracts which inhibits the formation of lactic acid in muscle.

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LIV. A FURTHER NOTE ON THE DETERMINATION OF IODINE IN BLOOD.

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In a recent paper [Aitken, 1930] a method of estimating iodine in blood was described, the essential feature of which was that the iodine was concentrated into a very small volume of solution before being titrated. The titration was performed with a 0·1 cc. pipette, and gave satisfactory results provided the blood was not abnormally low in iodine. However, it is evident that in the case of a blood containing say only 40 γ of iodine per kg. the error involved in the use of a 0·1 cc. pipette and $N/1000$ thiosulphate will be at least 5 %. In order to eliminate such an error the author has devised a convenient and accurate micro-burette in which the standard solution floats on a thread of mercury, the position of the latter being adjusted by means of a fine screw projecting into a mercury reservoir.

The burette, shown in Fig. 1, was made from the stem of a discarded 200° thermometer which had a capillary of internal diameter about 0·18 mm. One end of this was drawn out to a tip *H* and bent over at *G* as shown. The other end of the capillary was opened out slightly and sealed into the steel chamber *A* by means of cement. *B* represents a "stuffing-box" (also of steel) containing vaselined packing which is used to ensure that the chamber *A* is mercury-tight. The "stuffing-box" is screwed on to the chamber *A* which is threaded at the lower end for this purpose, the pitch of the screw being about 0·8 mm. The chamber *A* contains mercury as shown by the black shading. The lower end of the "stuffing-box" (but not of the chamber *A*) carries an internal thread to accommodate the fine steel screw *D*, which is turned by means of the milled head *E* and serves to control the level of the mercury in the thermometer capillary. The pitch of this screw is 0·33 mm. and its diameter 1·4 mm., so that one complete turn of the milled head *E* expels about 0·0005 cc. of mercury from the chamber, corresponding to about 10 divisions on the stem. As regards the other dimensions of the various parts, they may

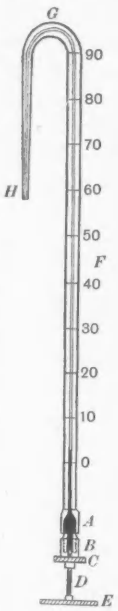

Fig. 1.

be gathered from the figure if it is remembered that in the actual apparatus the 10° graduations are 2 cm. apart.

The micro-burette, after being filled with mercury (which was done by filling the upper part of the "stuffing-box" with mercury and then attaching it to the chamber), was very carefully calibrated by drawing in threads of mercury at *H*, measuring their lengths in scale divisions and then weighing the threads. A table of corrections for variations in the diameter of the capillary was also drawn up, though these corrections were really negligible. The capacity of the burette showed that *N*/100 thiosulphate would be the most convenient standard to use, in which case 20 divisions on the scale corresponded to 0·21 γ of iodine in the sample.

The burette is filled with standard solution by immersing the tip *H* in a small vessel of the solution and rotating the milled head *E* forward until a small globule of mercury forms at the orifice; the head is then reversed and the standard solution drawn in to any convenient level. The tip is rinsed with distilled water, dried lightly with filter-paper and immersed in the solution in the titration-tube, and the titration is carried out by turning the milled head. Very delicate control is thus obtained as it is possible to raise the mercury level slowly through fractions of a scale division. In practice both the burette and the titration vessel are clamped to a burette stand, the whole being shaken bodily to ensure mixing during titration. The smallness of error arising in the burette can be gathered from the fact that it is easy to read to a quarter of a scale division on the stem and, using *N*/100 thiosulphate, this corresponds to 0·00262 γ of iodine in the sample.

Apart, however, from inaccuracies in measurement, some investigators have found that a large error arises in estimations based on Fellenberg's method on account of the incompleteness of the alcoholic extraction of the potassium iodide. Thus Andrew [1930] finds it necessary to multiply his experimental results by 3/2, while Adolph and Shen-Chao Chen [1930] use the factor 100/85. Andrew [1930, p. 273] finds that there is little or no loss involved in the first alcoholic extraction, while there is a considerable loss in the second. This discrepancy, though perhaps partly due to losses by volatilisation, is probably mainly to be accounted for by differences in the conditions under which the extractions were performed. The present author has had no difficulty in obtaining a quantitative recovery of iodide, provided there is sufficient carbonate present to provide a paste which can be properly treated. In recent work he has found it convenient when performing the second alcoholic extraction to imitate exactly the conditions of the first alcoholic extraction. Small nickel crucibles (about 25 cc. capacity) are used instead of platinum dishes. To the first alcoholic extract in these crucibles is added about 0·4 cc. of the potassium carbonate solution and the residue after evaporation is incinerated, the small crucible being placed in a slightly larger one to avoid the risk of overheating. The residue is carefully moistened with carbonate solution so as to form a paste when rubbed with two small glass rods

flattened at the ends, and successive small amounts of alcohol are employed for the extraction. Thus the only difference between the extractions is one of scale, and it is possible to perform them with equal completeness.

The author wishes to express his indebtedness to Dr C. E. Hercus for the interest he has shown in this work.

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LV. THE COLORIMETRIC DETERMINATION OF SODIUM.

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No easy, rapid and accurate method of determining sodium in foodstuffs and other biological material is at present available. Such a method should preferably be applicable to the same hydrochloric acid extract of the incinerated material in which calcium, magnesium and potassium are being determined. Many of the published methods are gravimetric [Kramer and Tisdall, 1921] and therefore are not only slow, but require relatively large amounts. Doisy and Bell's [1920] caesium method admittedly presents considerable technical difficulties. Of the other methods examined by us the most promising appeared to be those depending on the precipitation of the sodium as the sodium uranyl zinc (or magnesium) acetate [Barber and Kolthoff, 1928; Caley and Foulk, 1929], and the subsequent estimation of the uranium in the precipitate, either by reduction and titration with potassium permanganate, or colorimetrically with potassium ferrocyanide. Barrensheen and Messiner [1927] have described a colorimetric method based on this principle suitable for serum or other biological fluids, but, as described by them, the method is very unsatisfactory. If, for example, the precipitate is washed according to their directions it goes completely into solution during the second washing. Poulsson [1928] modified the method to overcome this obvious defect, but the method is still not directly applicable to acid extracts of incinerated materials. We have therefore subjected the whole technique to a critical study, and have established a method which can be applied to neutral or acid solutions of sodium salts. A further communication will shortly be made on the application of the method to blood and serum without incineration.

Principle.

This depends on the removal of free acids and phosphates with zinc acetate and hydroxide in 50 % alcohol. The sodium is next precipitated as the triple acetate with uranium and zinc. The uranium in the precipitate is estimated colorimetrically with potassium ferrocyanide. The standard colour is obtained either by submitting a standard solution of sodium to the same treatment as the unknown, or by using a standard solution of the triple acetate.

Reagents.

(1) *Alcoholic zinc acetate with zinc hydroxide.* As all the samples of zinc acetate we have examined contained small amounts of sodium we prepare the acetate as follows. To a hot strong solution of A.R. zinc sulphate add a slight excess of ammonia (sp. gr. 0·880). Filter on a Büchner funnel, wash thoroughly with hot water and finally suck as dry as possible. To 12·5 cc. of glacial acetic acid add the zinc hydroxide paste, prepared as above, in small amounts at a time until in slight excess. Filter and wash; make up the combined filtrate and washings to 100 cc.; add 3·0 cc. of ammonia (sp. gr. 0·880) and 300 cc. of 95 % alcohol.

(2) *Alcoholic uranyl zinc acetate reagent* [modified from Kolthoff, 1927]. (*a*) Dissolve 10 g. of uranyl acetate in 50 cc. of boiling water containing 2·0 cc. of glacial acetic acid. (*b*) Dissolve 30 g. of zinc acetate in 50 cc. of boiling water containing 1 cc. of glacial acetic acid.

Mix both solutions while boiling, raise the temperature again just to boiling, allow to stand overnight and filter. Mix the filtrate with an equal volume of absolute alcohol, allow to stand 48 hours at 0° and filter at 0°. The reagent is stable at room temperature.

(3) *95 % alcohol saturated with the triple acetate.* Prepare a sample of sodium uranyl zinc acetate by adding the uranyl zinc acetate reagent to some NaCl dissolved in 50 % alcohol. Filter or centrifuge and wash the precipitate thoroughly with 95 % alcohol. Suspend the precipitate in 95 % alcohol and allow to settle in the ice-chest. Use the supernatant fluid for washing the precipitate. Filter before use if not absolutely clear.

(4) *20 % potassium ferrocyanide.*

(5) *Standard sodium chloride.* Dissolve 1 g. of pure dry NaCl in water and make up to 100 cc. This forms the stock solution. For use dilute 2 cc. to 100 cc. 1 cc. of this dilute standard contains 0·2 mg. NaCl (0·0786 mg. Na).

(6) *Standard triple acetate.* (*a*) *Preparation of the stock solution.* Take 10 cc. of a 1 % solution of NaCl, add 80 cc. of water and 100 cc. of alcohol. Add 100–120 cc. of zinc uranyl acetate reagent. Stand for at least an hour. Collect this precipitate quantitatively and wash it carefully with ice-cold 95 % alcohol. Dissolve the precipitate in water and make up to 1000 cc. This forms the strong stock solution. Dilute some of this accurately 1 to 5. Take 5 cc. of this weak solution, dilute with water in a 25 cc. flask. Add 1 drop of glacial acetic acid and 0·5 cc. 20 % potassium ferrocyanide. Make up to 25 cc. The resulting colour is close to that obtained from 0·2 mg. NaCl (0·0786 mg. Na) in 2 cc. of water submitted to all stages of the method and made up to 25 cc.

In the removal of phosphate it is necessary to add alcohol to the aqueous solution containing Na originally measured, and to take an aliquot portion of the mixture. Since there is a contraction on mixing alcohol and aqueous

solutions the exact value of this aliquot is not known and therefore this triple acetate solution must now be accurately standardised against Na solutions.

(*b*) *Standardisation of the solution.* This should be done at least in quadruplicate. Take four samples of exactly 0·2 mg. NaCl (0·0786 mg. Na) in 2 cc. of water and subject them to every stage of the estimation, including the steps for the removal of phosphates. Transfer the precipitates to 25 cc. flasks. In two other flasks take 5 cc. of the dilute standard and add water to about 18–20 cc. Develop the uranium colour as described below in all six flasks. Match both standards against each of the quadruplicate flasks, setting the latter at 20 mm. Take the mean (the individual readings should all agree within 3 %). Suppose this to be 23 mm. The simplest method of using the standard is now to set it always at 23 mm. in the colorimeter. This is equivalent to the colour obtained by estimation from 0·0786 mg. Na, the precipitate being made up to 25 cc. and the colorimeter set at 20 mm. and the results must be calculated on this basis (see below). Those who prefer it may make the necessary dilution of the stock solution so that 5 or 10 cc. of the weak standard diluted to 25 cc. gives exactly the same colour intensity as that obtained from 0·0786 mg. Na.

Detailed description of the procedure.

In a centrifuge tube take an amount of the unknown solution containing 0·04–0·16 mg. of sodium. (This may be described as the normal range of the method, but as shown below the method will estimate accurately and directly amounts up to 0·8 mg. Na.) Dilute to 2 cc. with water, add 4 cc. of the alcoholic zinc acetate reagent, stir and cover with a rubber cap (10 cc. vaccine caps are suitable). Allow to stand for 2–3 hours at room temperature and leave at 0° overnight. While still cold centrifuge and take 3 cc. of the supernatant liquid into another centrifuge tube. Add 4 cc. of the uranyl zinc acetate reagent and stir with a glass rod drawn out at its end to about 1 mm. thick. The stirring should be continued until the precipitate begins to appear. Cover with a rubber cap and allow to stand for one hour at 0°. Centrifuge, pour off the liquid and drain the tubes by inverting on filter-paper; wipe the mouths of the tubes, and wash once with 5 cc. of the ice-cold alcohol saturated with the precipitate, taking care that the whole of the inside of the tube is rinsed. The precipitate should be stirred up. Centrifuge, and drain again. Dissolve the precipitate in water and transfer it quantitatively to a 25 cc. volumetric flask. If the precipitate is very bulky, indicating that the unknown contained more than 0·15 mg. Na, transfer it to a 50, 100, or even 200 cc. flask according to discretion. After a little experience it is easy to judge the size of flask required from the bulk of the precipitate. For the standard either take 1 cc. of the dilute sodium chloride solution (containing 0·2 mg. NaCl per cc.) and treat it in exactly the same way as the unknown and transfer it to a 25 cc. flask; or, take 5 cc. of the dilute standard triple acetate solution in a 25 cc. flask. To both standard and unknown add 1 drop of glacial acetic acid and

0·5 cc. of 20 % potassium ferrocyanide, make up to the mark with water, allow to stand for 3 minutes and match. The amounts of acetic acid and potassium ferrocyanide are those required for 25 cc. flasks; if larger flasks are used, proportionately larger amounts must be taken.

Calculation.

(*a*) *Using a standard NaCl solution.*

$$\text{Na mg. per 100 cc.} = \frac{20\ (\text{standard colorimeter reading})}{\text{Reading of unknown}} \times 0{\cdot}0786 \times \frac{100}{\text{Volume of unknown taken}}.$$

(*b*) *Using the standardised triple acetate solution.* Suppose the colorimeter set at 23 mm. (see p. 451) is equivalent to 0·2 mg. of NaCl submitted to all stages of the estimation made up to 25 cc. and set in the colorimeter at 20 mm. Then, although the arbitrary standard was set at 23 mm., the calculation is:

$$\text{Na mg. per 100 cc.} = \frac{20}{\text{Unknown}} \times 0{\cdot}0786 \times \frac{100}{\text{Vol. of unknown taken}}.$$

If 50 cc. or larger flasks have been used instead of 25 cc., the requisite additional factor must be introduced.

Discussion.

1. *Effect of* (*a*) *Time, and*
   (*b*) *Temperature on the precipitation.*

(*a*) Working with the same reagent, but in aqueous solution, Barber and Kolthoff [1928] found that precipitation was complete in ½ hour and directed that the mixture be left for 1 hour. We have shown that with our reagent 1 hour is sufficient to give complete precipitation.

Table I. *0·0786 mg. Na taken for estimation.*

Recovered at 0° (mg.)	
After 1 hour	After 18 hours
0·0785	0·0785
0·0782	0·0774
0·0793	0·0800
0·0762	0·0786

(*b*) We have found that from 0° to 10° the precipitation of sodium is complete, but that at 20° the precipitate is appreciably soluble and at 37°

Table II. *0·0786 mg. Na taken for estimation.*

	% recovered at			
	0°	10°	20°	37°
	100·0	100·5	92·7	79·6
	100·5	98·0	95·0	81·6
	99·5	99·2	92·7	80·3
	100·0	100·5	92·1	84·6
Mean	100·0	99·8	93·0	81·5

much more so. The precipitation therefore should be carried out at 0°. Table II shows the extent of the error introduced.

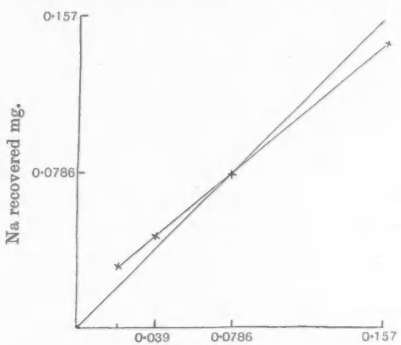
2. We have satisfied ourselves by direct experiment that the intensity of the colour developed by the potassium ferrocyanide is directly proportional to the amount of the triple acetate present. From a stock solution of the triple acetate 2 cc. of which contained about 0·039 mg. Na the following results were obtained: 1 cc., 2 cc. and 4 cc. were measured into 25 cc. flasks each in quadruplicate, 1 drop of glacial acetic acid and 0·5 cc. of potassium ferrocyanide were added to each and water added to make up to 25 cc. The flasks containing 2 cc. of stock triple acetate solution were taken as standard; they agreed with each other. The standard was then set at 20 mm. in the colorimeter and the other solutions matched against it. The results are shown in Table III.

Table III.

cc. triple acetate taken	Actual colorimeter reading mm.	Theoretical colorimeter reading mm.
1	39·4	40
1	39·4	40
1	41·0	40
1	40·5	40
4	10·0	10
4	10·1	10
4	10·0	10
4	10·0	10

The range of colour comparison is therefore correct for intensities from half to twice that of the standard.

3. When we first used this method we followed Kolthoff's [1927] directions for making up the precipitating reagent and did not add an equal volume of alcohol. On testing the method by taking known amounts of sodium we



Theoretical curve ———. Experimental curve × ——— ×.
The colour developed from 0·0786 mg. Na is taken as standard and all the other values expressed as fractions of this.

Fig. 1.

invariably obtained the results shown in Fig. 1. Such a curve would have necessitated working with the colour of the unknown very close to that of the standard and would therefore have greatly restricted the range of the method.

This deviation from the theoretical recovery would be accounted for by the presence of 0·017 mg. of sodium as impurity in both the unknown and the standard. We had great difficulty in locating this impurity. Alcohol, distilled water and acetic acid were all suspected in turn, but the contaminating sodium was finally found in solution in the actual reagent used to precipitate the sodium. In the absence of alcohol some, or all, of the sodium present originally as an impurity in the zinc acetate remains in solution, and precipitates on adding the reagent to the alcoholic solution resulting from the removal of phosphates. After modifying the reagent the recoveries became theoretical (see Table IV below).

4. *Range of the method.* The amount of sodium precipitated by 4 cc. of reagent from the 2 cc. of aqueous solution taken is strictly proportional to the amount of sodium present from 0·02 to 0·8 mg. The larger amounts must be made up in larger flasks and the smallest made up to 8 cc. in the centrifuge tube without transference (see below under potassium interference).

Table IV.

mg. NaCl taken	Recovered	% recovery	mg. NaCl taken	Recovered	% recovery
0·05	0·0482	96·3	0·2	0·204	102
0·05	0·0502	100·5	0·2	0·200	100
0·05	0·0516	103·0	0·2	0·200	100
0·05	0·0500	100·0	0·2	0·202	101
0·1	0·097	97	0·4	0·405	101·3
0·1	0·100	100	0·4	0·405	101·3
0·1	0·100	100	0·4	0·416	104
0·1	0·098	98	0·4	0·412	103
0·2	0·192	96	2·0	2·05	102·5
0·2	0·195	97·5	2·0	1·94	97
0·2	0·195	97·5	2·0	2·04	102·0
0·2	0·206	103	2·0	1·96	98

Note. All the determinations were carried out against an arbitrary standard.

5. *The standard colour.* Although much easier to prepare, a solution of uranium acetate does not make such a satisfactory permanent standard as a solution of the triple acetate. This is because the zinc present in triple acetate solutions begins to precipitate as the colloidal ferrocyanide after some minutes. If the colour developed from triple acetate is compared in the colorimeter with that from a uranium acetate solution it will be found that the triple acetate colour remains stable for about 10 minutes and then appears slowly to become more intense. This increase is accompanied by the appearance of a colloidal precipitate which can only be seen when the solution is viewed by reflected light. The change, however, is slow and we have used a uranium acetate standard quite satisfactorily by matching always between 3 and 8 minutes after developing the colour. All these difficulties are overcome by

using a solution of the triple acetate as standard. The colour comparisons are then stable for at least an hour, but we advise matching within 20 minutes of the development of the colour. The standardisation of this arbitrary standard has already been explained in detail under "Reagents."

6. *Interfering substances.* Caley and Foulk [1929] have shown that Ca, Mg, Sr, Ba and Fe do not interfere with this estimation of sodium. We can confirm their statement that Ca, Mg and Fe do not interfere. We have not tested Sr and Ba. The same authors report that lithium interferes with the precipitation, but there is practically no lithium in biological materials.

Phosphates are well known to be precipitated by uranium and must be removed. Table V shows that this is effectually accomplished by the zinc acetate in 50 % alcohol. A solution was prepared containing 9·82 mg. Na (25 mg. NaCl), 19·5 mg. K, 1·0 mg. Ca, 2·0 mg. Mg, 0·1 mg. Fe and 15·5 mg. P per 100 cc. The amount of sodium present in 1 cc. was estimated in quadruplicate. 1 cc. of a pure solution of NaCl containing 0·0786 mg. Na per cc. was also estimated in quadruplicate. All the colours were matched against an arbitrary triple acetate standard. The recoveries are given in Table V.

Table V.

Taken	Recovered mg.	%
1 cc. pure NaCl = 0·20 mg. NaCl	0·201	100·5
" "	0·203	101·5
" "	0·199	99·5
" "	0·199	99·5
1 cc. salt mixture = 0·25 mg. NaCl	0·248	99·3
" "	0·243	97·4
" "	0·248	99·3
" "	0·251	100·5

Arsenates also are stated by Barber and Kolthoff [1929] to interfere, but we have not deemed it necessary to investigate their removal as they are absent from most biological material.

As potassium is often present in considerable excess over sodium in biological material, we have tested the effects of large amounts and have found that the method is satisfactory if the amount of potassium present in the volume taken for analysis does not exceed 0·6 mg. Table VI shows that interference is definite in the presence of 0·9 mg. K.

Table VI. *0·0786 mg. Na taken.*

% recovered in the presence of	
0·62 mg. K	0·93 mg. K
97·0	120·5
100·7	119·2
100·0	119·2

0·6 mg. K is about 8 times the amount of Na used for the standard. In vegetable materials, *e.g.* grass, this ratio is often exceeded. When this is so the following modification of the method should be employed. Dilute the

specimen until 2 cc. contains less than 0·6 mg. K. Proceed as already described till the washing of the precipitate is completed. Now evaporate the residual drop of alcohol on a water-bath. Add 7·5 cc. of water and 0·5 cc. of 7 % potassium ferrocyanide acidified slightly with acetic acid. Match against the same standard that is always used. By this means it is possible to work with K/Na ratios of 32:1.

7. *Attention to detail.* Scrupulous care is essential when determining small amounts of Na. The element is ubiquitous so that the chances of contaminating the glassware and reagents are manifold. The authors recommend that the reagents be tested for purity from time to time in the following simple way. Take 0·04 and 0·08 mg. Na (or 0·1 and 0·2 mg. NaCl), make each up to 2 cc. and submit these to every stage of the sodium estimation. Make up the precipitates to 25 cc. Develop the colours and set the stronger at 20. The weaker should read 40. If it reads less than 40 one or more of the reagents contains sodium.

Summary.

1. A method is described by which 0·02–0·8 mg. Na may be directly determined. In the absence of phosphates the range is 0·01–0·4 mg. Na.

2. The sodium is precipitated as sodium uranyl zinc acetate and the uranium is determined colorimetrically with potassium ferrocyanide.

3. The method is applicable to neutral or acid solutions.

4. Ca, Mg and Fe do not interfere. Phosphates interfere, but are removed with zinc acetate.

5. Sodium may be determined directly in the presence of 30 times its weight of potassium.

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LVI. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

II. VARIATIONS IN THE SENSITIVITY TOWARDS INSULIN OF DIFFERENT SPECIES OF ANIMALS ON CARBOHYDRATE-FREE DIETS.

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In each set of experiments described in our previous communication [Hynd and Rotter, 1930], a number of animals were tested for their response to insulin. It was thus possible to compare the effects produced by an injection of the hormone not only with regard to the previous dietetic treatment of the animal, but also in relation to the existing blood-sugar level, the muscle-glycogen content, and the distribution of glycogen and fat in the liver.

All the experiments were conducted in a room the temperature of which was maintained at 20–22°, for although the symptoms develop less rapidly at this temperature than at higher temperatures such as 30–37°, as first shown by Krogh, our conditions are more normal and hence less likely to bring other factors into operation. The same sample of insulin was used throughout the experiments recorded in this paper, and the injections were made subcutaneously. The dose employed for the several species varied slightly, the amount used being that found necessary to produce in control animals the train of symptoms accompanied by what are usually referred to as "hypoglycaemic convulsions."

Experiments with mice.

For the experiments with mice the insulin was diluted 1 in 10 with 0·05 % hydrochloric acid, 7 minims of this diluted insulin being the dose for a mouse of 20 g. body weight. As the diets employed have already been described [Hynd and Rotter, 1930], it is only necessary to indicate the number of animals employed in the several experiments. On carbohydrate-free diets 41 mice were tested for their response to insulin, namely 15 on cheese, 14 on butter-fat, and 12 on caseinogen, and these tests were controlled by 23 animals fed on a normal diet.

With one exception all the mice on the control diet showed the characteristic insulin symptoms between 1 and 1½ hours after the injection, and re-

quired to be resuscitated by the administration of glucose or of maltose. The cheese-fed mice, on the other hand, were without exception resistant to the same dose of insulin, and, even with twice the dose, the only effect of the injection of insulin was to render the animals somewhat quiet and drowsy. All returned to normal in 2–3 hours without treatment of any kind. To elicit severe symptoms including convulsions, the dose required to be increased 3–4 times. Cheese-fed mice, which had been proved resistant to insulin, showed a normal response after 12 days' subsequent feeding on oats, marked lowering of the blood-sugar (0·062) occurring in 30 minutes after the insulin injection and the convulsive level (0·054) being reached in 65 minutes.

Similar results were obtained with mice reared on caseinogen until 7 weeks old, although one animal died 30 minutes after being injected. Of the remaining 11 mice used, 7 showed no symptoms except drowsiness, and the other 4, in addition to being drowsy, showed slight sprawling of the limbs. This, however, did not develop further and the animals were normal in the course of 3 hours. Mice rendered resistant to insulin by a diet of caseinogen remained so, though supplied for 5 days with as much (*a*) glucose, or (*b*) fructose, as they would take in addition to the usual amount of caseinogen. These experiments were not continued further, as the animals refused after a time to eat the pure sugars.

Of the 14 fat-fed mice, 6 showed no symptoms except drowsiness, 6 others became slightly sprawling for a time, and only 2 showed severe symptoms requiring glucose injection to bring about recovery.

Evidently either for maintaining the growth and health of the animals, or for producing resistance to the effects of insulin, a carbohydrate-free diet, consisting exclusively of either fat or protein, is not so satisfactory as one in which both protein and fat are present. Nevertheless, on all the carbohydrate-free diets employed, mice showed a marked difference in their sensitivity towards insulin from animals on a normal diet. After 2 weeks' feeding on a carbohydrate-free diet, resistance to insulin is well established, and the mice were still resistant when the cheese diet had been continued for $2\frac{1}{2}$ months.

Experiments with rats.

From the experience gained in feeding carbohydrate-free diets to mice, only two diets were attempted with rats, namely a carbohydrate-rich diet of bread and milk, and a carbohydrate-free diet of cheese. Twenty-two animals on each of these diets have been tested with insulin, the dose being proportionately less than that given to mice, namely 10 minims of 1 in 5 insulin per 100 g. of body weight. As we found that the rate at which hypoglycaemic symptoms developed in a normal rat seemed to depend, in part at least, on the strength of the insulin solution injected, we have used invariably for the experiments with rats 1 part of insulin diluted with 4 parts of 0·05 % HCl. Also as piebald rats are somewhat variable in their response to insulin, all the experiments here reported were carried out on albinos.

Under the dosage indicated above, rats on the cheese diet showed no notable symptoms except drowsiness, while the control rats without exception had severe convulsions after about 2 hours, and required to be relieved by the injection of glucose.

The following is typical of the different behaviour of the two sets of rats.

	Rat, ♂, 161 g. Diet of bread and milk	Rat, ♂, 160 g. Diet of cheese for 14 days
9.43 a.m.	Injected with 16 minims of 1:5 insulin	Injected with 16 minims of 1:5 insulin
11.0 „	Not quite normal: climbs wire cage on being disturbed	Not quite normal: remains quiet
11.5 „	Irritable; no sprawling	Legs sprawling; feels rigid when handled; no fall of body temperature; not irritable
11.15 „	Symptoms developing	Improving; looks drowsy; otherwise almost normal
11.45 „	Sprawling limbs; cold and limp	Quiet and drowsy; otherwise normal
11.51 „	Severe convulsion	Drowsy
11.57 „	Severe convulsions	Drowsy
12.1 p.m.	Severe convulsion	Drowsy
12.5 „	Another convulsion	No further symptoms developed
12.10 „	Injected 1 cc. 20 % glucose solution	Taking food (cheese moistened with milk)

In the experiments with rats, it has been noticed that almost invariably, previous to the stage when symptoms develop in the carbohydrate-fed animals, the cheese-fed rats appear somewhat worse than the controls. They are not so irritable, but much less active, and show slight sprawling of the hind limbs. However, they do not become cold and when handled appear rigid rather than limp. This effect lasts only a very short time, and the animals then improve without further symptoms developing. If the dose of insulin has been too large, or the carbohydrate-free diet has not been continued long enough, mild insulin symptoms may develop, but if so, they appear later than in the control rats, as noted by Bainbridge [1925].

As we have already shown that the livers of cheese-fed rats have a maximum fat content after a fortnight's feeding, experiments were carried out to determine (1) whether the resistance to insulin is also at a maximum at the same time, and (2) when resistance first develops. The results obtained were inconclusive. Partial resistance to insulin could be detected after 4 days on the cheese diet, as after this period of feeding the symptoms were delayed in development and less severe than those of the control animals. After 8–10 days, resistance was well established, and was still well marked after 1 month. This is in agreement with the observations of Abderhalden and Wertheimer [1924, 1], and of Bainbridge [1925]. The former workers noted resistance in rats after 10 days' feeding, while the latter employed periods of 2–6 weeks' feeding. In our experiments, all animals after 14 days' feeding on cheese were resistant to insulin. It was also found that, after the withdrawal of food for 24 hours, rats which had been fed on cheese for a fortnight were still resistant to the effects of insulin.

Experiments with rabbits.

Only one experiment was carried out on rabbits, in which we confirmed the observation made by several workers (Page [1923], Blatherwick *et al.* [1924], Abderhalden and Wertheimer [1924, 3], Pénau and Simonnet [1925], Geiger and Kropf [1929]), that rabbits on green food are less resistant to insulin than animals fed on oats. The object of our experiment, however, was not merely to confirm this behaviour of rabbits, but rather to determine whether any departure from the normal could be detected in the respective reducing power of the blood-plasma and corpuscles of such animals. As is evident from the results tabulated below, no significant differences were obtained on the two types of diet.

Diet	Whole blood-sugar %	Plasma-sugar %	Corpuscle-sugar %	Plasma volume	:	Corpuscle volume
Greens	0·113	0·144	0·047	76·66	:	23·34
Oats	0·134	0·147	0·060	77·71	:	22·29

Experiments with kittens.

As we had found previously that kittens were more responsive than rabbits to the same dose of insulin, it seemed desirable to extend our observations to this species. The carbohydrate diet was again bread and milk, while the carbohydrate-free diets employed were (*a*) white fish, and (*b*) cream, the former consisting largely of protein, the latter having a high fat content, and both suited to the type of animal under investigation. Four sets of experiments have been carried out. In each set the kittens employed were of the same litter and were 6 weeks old at the commencement of the experiment. After 3–4 weeks on the diets indicated above, they were injected subcutaneously with 20 minims of insulin per kg. of body weight.

The kittens on all three diets responded to the injection and displayed the characteristic symptoms, including leg-weakness, mewing, salivation and convulsions [Olmsted and Logan, 1923]. Relief followed the administration of glucose, but there were frequent relapses. Hence a smaller dose of insulin, namely 10 minims per kg., was tried, but with similar results. Typical symptoms ensued in all cases, and the only difference noted was the length of time required for the symptoms to develop. The time of onset of the first convulsion in each case is summarised in the following table, from which it is seen that the animals on the carbohydrate-free diets were always much earlier in showing symptoms than the carbohydrate-fed animals. In fact, on two occasions the kittens fed on bread and milk did not have convulsions.

	Period which elapsed before first convulsion (minutes)			
Diet	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Fish	60	75	75	75
Cream	80	80	90	—
Bread	180	(No convulsion)	(No convulsion)	105
Minims of insulin	20	10	10	10

Discussion.

On a carbohydrate-free diet rats and mice, while showing no significant change in muscle-glycogen when compared with animals on a carbohydrate-rich diet, tend to have a high blood-sugar, an increased liver-fat content, and a diminished liver-glycogen [Hynd and Rotter, 1930]. Can any of these differences be correlated with the resistance to insulin that such animals develop when maintained on a carbohydrate-free diet?

In the table which follows are summarised the blood-sugar levels of mice on different diets and their conditions at varying periods after the injection of insulin. On the control diet of bread and milk, the blood-sugar is seen to fall rapidly and the convulsive level is soon reached. On the carbohydrate-free diets, on the other hand, though the actual range through which the blood-sugar is lowered by insulin is approximately the same, the change takes

Experiments with mice.

	Without being fasted						Fasted 18 hours			
Diet ...	Bread		Cheese		Fat		Bread		Cheese	
Time after insulin injection (mins.)	Blood-sugar %	Condition of animal	Blood-sugar %	Condition of animal	Blood-sugar %	Condition of animal	Blood-sugar %	Condition of animal	Blood-sugar %	Condition of animal
0	0·124	Normal	0·157	Normal	0·191	Normal	0·174	Normal	0·171	Normal
30–40	0·05	Very irritable	0·087	,,	0·076	,,	0·087	Slightly affected	0·073	,,
50–60	0·039	Convulsion	—	,,	0·069	,,	0·056	Convulsion	—	,,
70–80	0·038	,,	0·060	,,	—	,,	—	—	—	—
90	0·039	,,	—	,,	0·049	,,	—	—	—	—
150	—	—	0·057	,,	0·056	,,	—	—	0·064	Slightly sprawling
240	—	—	0·131	,,	—	,,	—	—	0·064	Normal
Max. fall	0·086		0·100		0·142		0·118		0·107	

place much more slowly and the animals remain more or less normal. Further, as animals which have fasted 12–24 hours have their blood-sugars at the same level as controls, but are still resistant to insulin, it is not the initial high blood-sugar level but rather the diminution in the rate at which the fall in blood-sugar is produced that is concerned with the resistance to insulin. This is supported by the experiments with rats on carbohydrate-free diets carried out by Abderhalden and Wertheimer [1924, 2], who have reported that such animals after the injection of insulin display a less rapid fall in blood-sugar. Tiitso [1925] also found that the blood-sugar falls less rapidly in starved rabbits than in those fed with abundant carbohydrate.

Since the blood-sugar level represents the balance between two sets of opposing processes, it is necessary to inquire if we can discover any factor responsible for the diminished rate at which the blood-sugar falls in non-carbohydrate-fed rodents, since it appears that there is some connection between the diminution in rate of fall of blood-sugar and the response to insulin.

The difference in the effects of insulin on well-fed as compared with starved animals was considered by McCormick, Macleod, Noble and O'Brien [1923] to depend primarily upon the amount of liver-glycogen. Lawrence [1926], also, in emphasising the importance of the glycogen content, stated that when the glycogen stores were empty insulin was much more effective in its hypoglycaemic action than at any other time. Accordingly, as we have found that animals on a carbohydrate-free diet show a reduced liver-glycogen content, one would predict that such animals should respond more readily to an injection of insulin. Experiment, however, shows just the opposite result. Non-carbohydrate-fed rodents withstand insulin much better than those receiving abundant carbohydrate, both when judged by the rate of fall of the blood-sugar and the incidence of convulsions. The amount of glycogen stored in the liver, therefore, is not, in all circumstances, a reliable index of the sensitivity of an animal towards insulin. In other words, there is some factor more important than the available amount of stored glycogen.

Bainbridge [1925] believed resistance to be due to excess of fat rather than to absence of carbohydrate from the diet, and sought to establish a connection between resistance to insulin and the fat content of the diet. Our results do not support this contention. The livers of rats and of mice on a cheese diet show a high liver-fat content, which reaches a maximum at about that period when maximum resistance to insulin develops, but as the excess of liver-fat is extremely mobile and disappears after 24 hours' starvation, lack of response to insulin is not connected with the excessive amount of fat, for starved animals are still resistant to insulin. Moreover, we have succeeded in producing marked resistance by feeding mice on a purely protein diet, namely on caseinogen, while several workers have reported on the resistance of rabbits fed on diets containing little or no fat. Again, it is well recognised that animals on a high carbohydrate diet after a time become distinctly fatty. Yet such fattened animals do not show resistance to insulin. On the other hand, the absence of carbohydrate from the diet was considered by Abderhalden and Wertheimer [1924, 1, 2] to be a factor of importance in establishing resistance to insulin. Some support for this opinion is found if one accepts Laufberger's [1924] view that the essence of insulin action is to inhibit gluconeogenesis, for when no preformed carbohydrate is supplied, the formation of carbohydrate from other substances must of necessity become accentuated, and consequently a diminished insulin effect would result in animals on a carbohydrate-free diet. However, the investigations of Grevenstuk, de Jongh and Laqueur [1925] indicated that the resistance was not due to any specific type of foodstuff, but depended on the relative proportions of carbohydrate, protein and fat.

Generally speaking our observations on the resistance of rodents to insulin are in agreement with those recorded by other workers: and it is quite clear that neither the stores of glycogen nor of fat are responsible for the resistance to insulin. But a further difficulty arises when the results of the experiments

with kittens are considered, because in this type of animal neither a diet consisting entirely of protein, nor one containing a large amount of fat, brought about resistance to insulin. In fact, our experiments showed that kittens on a carbohydrate-rich diet were somewhat less responsive to insulin than animals on carbohydrate-free diets. However, the carbohydrate-fed kittens could not be described as resistant to insulin, as, though in two cases no convulsions occurred, the animals were definitely affected. Had a smaller dose of insulin been given, resistance might have been claimed, but the chief point for the present is that kittens differ from mice and rats in that feeding a carbohydrate-free diet does not render them resistant to the action of insulin.

Eadie [1930] has recently reported a difference in the action of adrenaline in the cat and the rat, and Corkill [1930] found the young rabbit to differ from several other species with regard to the increase of liver-glycogen after insulin. Funk [1922], also, has pointed out that dietary changes have often an influence on the toxicity of certain drugs. Consequently we have here a parallel to our results with regard to the action of insulin. In other words, there is a distinct species difference not only between kittens, which may be taken as representative of carnivora, and rodents, but also amongst the rodents themselves.

Now it is well known that while the alkali reserve of a rabbit is easily changed, it is exceedingly difficult to alter that of a dog or a man, and hence probably also that of a cat [McClendon, 1919]. Feeding on oats was found by Page [1923] and also by Abderhalden and Wertheimer [1924, 3] to lower the alkali reserve of a rabbit, and a non-carbohydrate diet would be capable of a similar effect in the case of a mouse or a rat [Abderhalden and Wertheimer, 1925], but not in a dog, cat or man [Michalowsky, 1930]. We thus arrive at the reasonable conclusion that an animal when maintained on a diet which is capable of bringing about a lowering of its alkali reserve will tend to show resistance to the action of insulin. There is thus an element of truth in the views of both Abderhalden and Wertheimer [1924, 1, 2] and of Bainbridge [1925], already referred to, as either absence of carbohydrate or excess of fat inevitably leads to a diet tending to reduce the alkali reserve. Though certainly not the only factor, the acid-base equilibrium of the body is thus a fundamentally important one in the mechanism of insulin action, in that it probably regulates the effect not only of the internal secretion of the pancreas, but also the effects of the other ductless glands, such as the suprarenals, thyroid and pituitary, which are known to be concerned in carbohydrate metabolism. In a forthcoming paper further experiments will be described in support of this view.

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LVII. EFFECT OF THE ADDITION OF SALTS ON THE p_H OF SOLUTIONS OF AMPHOLYTES.

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It has been found that the velocity of hydrolysis of starch in the system gelatin-starch-amylase is affected by the addition of calcium chloride. This effect may be due to change in the hydrogen ion concentration of the system.

Such a possibility was first pointed out by Spiro [1922], who found that the p_H of glycine solutions was lowered by addition of calcium or potassium chloride, and similar results were obtained by Mond [1923], Netter [1925], Simms [1929], and others. The majority of these authors interpreted the result of addition of electrolytes to colloidal sols as being essentially the same as in the case of crystalloid ampholytes, such as glycine.

Such a view is not, in our opinion, sufficiently justified by the experimental material available, as the effects of relatively high concentrations (0·15–0·25 M) of electrolyte only were examined, and, further, the dependence of the observed phenomena on the concentration of electrolyte was not investigated. The work of Freundlich [1930] on hydrophobe, and of Kruyt [1928, 1929] on hydrophile, colloids has shown that the influence of salts on the electrolytic properties of colloidal particles should rather be evidenced by low (0·02 M) than by high concentrations of salt.

In the present paper, experiments on the change in p_H of solutions of gelatin, ovalbumin, peptone, and glycine will be described, in relation to the concentration within the limits 0–0·025 M of salt added, to the valency of the anion and cation of the salt and to the original p_H of the solution.

Experimental.

Gelatin (Coignet et Cie, Paris) was twice washed with cold distilled water, and dissolved at 60°. 100 cc. portions of 0·5–2·0 % solutions were placed in flasks containing 10 cc. of water or of various salt solutions, and the p_H of the systems was determined potentiometrically at 20°, using a hydrogen-calomel electrode. Special attention was paid to the purity of the water and to the cleanliness of the vessels used, since even traces of impurity might affect the colloidal state of the gelatin. The salts used were sodium, potassium,

calcium, and magnesium chlorides and sodium and magnesium sulphates (Merck's "chemisch rein" chemicals). The p_H of the salt solutions was brought to that of the gelatin solution by the addition of 0·02–0·1 *N* sodium hydroxide, which was also used for bringing the gelatin solution to the desired p_H.

Similar experiments were performed using Witte's peptone (Poulenc Fr.); this peptone contained colloidal matter precipitable by the addition of 50 % alcohol. The ovalbumin used (Merck) contained globulin, which, however, separated on standing and was removed by filtration.

I. *Gelatin.*

These experiments (Table I) were performed on 1 % solutions of gelatin, the p_H of which was 5·8–5·9. It appears that whilst the salts used act quantitatively differently, yet in all cases the p_H of the system is lowered. This is best illustrated by Table I, which shows that the depression of p_H is, except at very low concentrations, proportional to the concentration of potassium chloride. Bivalent anions and cations act differently, being much more active per unit of concentration when present in small (1–10*mM*) than in higher concentrations, and the curves obtained for such salts are of the type of adsorption isotherms.

Table I. *Variations in* p_H *of gelatin solutions.*

p_H of control systems = 5·8–5·9.

Molar concentration of salt	Lowering of p_H				
	$CaCl_2$	$MgCl_2$	$MgSO_4$	Na_2SO_4	KCl
0·0025	0·21	0·12	0·09	0·06	—
0·005	0·25	0·20	—	—	—
0·010	0·30	0·26	0·19	0·15	—
0·020	0·39	0·33	—	—	—
0·025	0·43	0·38	0·27	0·26	—
0·050	—	—	—	—	0·09
0·100	—	—	—	—	0·17

The results obtained in systems in which the concentration of calcium chloride is maintained constant whilst that of gelatin is varied (0·4–1·2 %) are given in Table II, in which we see that the depression in p_H caused by the salt is greater at lower than at higher concentrations of gelatin.

Table II. *Influence of concentration of gelatin.*

Gelatin %	Concentration of $CaCl_2$ *M*	Lowering of p_H
0·4	0·010	0·38
	0·020	0·59
0·6	0·010	0·48
	0·020	0·50
0·8	0·010	0·44
	0·020	0·46
0·9	0·020	0·44

The reaction of gelatin solutions was varied by adding 0·02 *N* sodium hydroxide solutions to the required p_H; in this way three solutions were prepared, of p_H 5·90, 8·03, and 9·21, from the same preparation of gelatin, and of the same concentration. The greatest depression of p_H due to the addition of electrolytes was found at p_H 8·03 (Table III), 0·005 *M* calcium chloride lowering the p_H by 0·46, whilst at p_H 5·90 (Table I) the observed depression is only 0·25, and at p_H 9·21 0·03; the corresponding depressions observed in the presence of 0·01 *M* salt are respectively 0·54, 0·30 and 0·05. The solution remained clear at p_H 5·90 and 8·03, whilst at p_H 9·21 opalescence was observed on the addition of calcium chloride. Substantially similar results were obtained with magnesium chloride in place of calcium chloride.

Table III. *Influence of original* p_H.

Concentration of $CaCl_2$ *M*	p_H	Lowering of p_H
0·0	8·03	0·0
0·005	7·57	0·46
0·010	7·49	0·54
0·0	7·44	0·0
0·0025	7·23	0·21
0·0	9·21	0·0
0·005	9·18	0·03
0·010	9·16	0·05
0·025	9·01	0·25

II. *Ovalbumin.*

These experiments were performed on 2 % aqueous solutions of ovalbumin which were filtered overnight through filter-paper. The effect of adding calcium chloride or magnesium sulphate to such solutions is given in Table IV; the curve connecting variation in p_H with concentration of electrolyte is also of the adsorption isotherm type.

Table IV. *Ovalbumin solutions.*

Salt concentration *M*		p_H	Depression of p_H
$CaCl_2$	0·0	8·58	0·0
,,	0·005	8·42	0·16
,,	0·010	8·37	0·21
,,	0·020	8·32	0·26
,,	0·025	8·31	0·27
$MgSO_4$	0·010	8·47	0·11

III. *Peptone.*

The action of various concentrations of calcium and magnesium chlorides, magnesium and sodium sulphates, and sodium chloride on 3 % peptone solutions at p_H 4·8 and 5·7–6·0, was examined. At the isoelectric point for peptone (p_H 4·8) it was found that electrolytes possessing a bivalent anion (sodium sulphate) or cation (magnesium or calcium chloride), or both together (magnesium sulphate) produced only a negligible depression of p_H (Table V).

Table V. *Peptone.*

Concentration *M*	Depression of p_H			
	$CaCl_2$	$MgCl_2$	$MgSO_4$	Na_2SO_4
Original p_H = 4·78–4·80:				
0·005	0·05	0·06	0·05	0·05
0·010	0·07	0·09	0·07	0·07
0·025	0·08	0·12	0·09	—
Original p_H = 5·8–6·0:				
0·010	0·23	—	0·17	—
0·025	0·42	0·38	0·38	—

Solutions brought to p_H 5·7–6·0 by the addition of sodium hydroxide exhibit, on the other hand, a marked depression of p_H on the addition of bivalent ions. The magnitude of the change in p_H is a function of the initial p_H of the solution.

IV. *Glycine.*

The results obtained (Table VI) are identical with those obtained by earlier workers.

Table VI. *Glycine.*

Concentration of $CaCl_2$ *M*	p_H of solution	Lowering of p_H
0	6·78	—
0·025	6·71	0·05
0·050	6·66	0·12
0·100	6·64	0·14

Discussion.

The results of the above experiments on the whole confirm those of earlier workers in this field, who found that the addition of salts depresses the p_H of solutions of amphoteric electrolytes, should this be above the isoelectric point. This phenomenon might be interpreted as being due to change in the activity factor, f_a, of the salts already present, according to the equation:

$$C_H = K\,[\text{acid}]/f_a\,[\text{salt of acid}].$$

This explanation does not, however, embrace the phenomena observed in their entirety. Thus, if we accept the existence of zwitterions, the dissociation not only of the carboxyl groups, but also that of part of the amino-groups has to be taken into consideration, when the action of added salts is the resultant of their action on both the acidic and the basic groups of the ampholyte. Next, apart from the activity factor, f_a, other factors, arising from the degree of dissociation of the salts added, may come into play, particularly in the case of salts giving rise to multivalent cations. Thirdly, at certain p_H values the salt may, as a result of hydrolytic dissociation, give rise to hydroxides possessing a smaller f_a than that of the base added to render the solution alkaline. Finally, the solubility of the salts formed with the ampholyte should

be taken into consideration; thus at certain p_H values the addition of calcium or magnesium chloride to ovalbumin solutions gives rise to turbidity.

We shall in a future communication examine more closely the factors capable of reducing the p_H of ampholyte solutions on addition of electrolytes; in this paper we desire to draw attention to the following facts.

The results obtained by adding different concentrations of electrolytes (0·0025–0·025 M) are very different from those observed in solutions of crystalloids. Michaelis and Krüger's [1921] experiments on the effect of salts on the p_H of solutions of phosphoric acid, glycine, *etc.*, have shown clearly that, within the limits of concentration of salt studied, these changes are quite inconsiderable and are proportional to the concentration of salt added. Our experiments show on the other hand that salts giving rise to bivalent cations or anions provoke, within certain p_H limits, considerable change in p_H, which is not proportional to the concentration of salt added. Thus, using 1 % gelatin solution at an initial p_H 5·9, the addition of calcium chloride to a concentration of 0·0025 M lowers the p_H by 0·21, the addition of a second portion of salt to 0·005 M lowers the p_H by only 0·04, whilst on addition of a third portion the difference is barely 0·025. Substantially the same results are obtained using magnesium chloride (first portion 0·12, second 0·08, third 0·03) or sulphate or sodium sulphate. This phenomenon is least marked with potassium chloride, in which case a concentration of 0·1 M, *i.e.* 40 times as great as for calcium chloride, has to be employed in order to achieve the same effect.

It will readily be seen from the above that the results obtained are quite different from those found for glycine, addition to which of calcium chloride to 0·0025 M reduces the p_H by only 0·02.

The reason for the different behaviour of univalent and bivalent ions may lie in the greater effect of the latter on the activity factor of the ampholyte, as follows from Eucken's [1930] formula:

$$\log f_a = - 0{\cdot}354 n_i^2 \sqrt{\Gamma},$$

where $\Gamma = \Sigma c_i n_i^2$, c_i is the ionic concentration, and n_i is the charge on the ions; the term n_i occurs twice.

A second reason for this difference may be differential aggregation of ions on the surface of the colloid, as a result of adsorption. The researches of Freundlich and of his collaborators led him to the conclusion that, even assuming equal adsorption of uni-, bi- and ter-valent ions, the effect will be the greater the higher the valency of the ions. It appears to us that the fact that the valency factor occurs twice in the formula expressing the activity factor can only partially explain the difference in the effect of varying the concentration of potassium chloride on the one hand and of calcium and magnesium chlorides and of sodium and magnesium sulphates on the other.

It is our opinion that the difference in the action of salts on the p_H of colloidal solutions of gelatin, ovalbumin, and peptone, in comparison with

their action in the case of crystalloids, is a consequence of the sorption of ions on the surfaces of the colloidal particles. This view is supported by the shape of the curves obtained, which resemble adsorption isotherms. The ions undergoing sorption are concentrated in the hydrosphere of the colloidal particles; since it is in this region that compounds responsible for the [illegible] the solution, such as albuminates, are present, it would follow that the [illegible] of added salts should be greater in heterogeneous than in homog[illegible] systems. Should it be desired in such cases to determine $\log f_a$ a[illegible] would be necessary to substitute for C_i, the total ionic concentration [illegible]nit volume, not the concentration calculated for unit volume of the syst[illegible]m as a whole, but for unit volume of the hydrosphere of the colloidal particles.

The differential actions of various electrolytes would be a co[illegible]sequence of the different valencies of their ions, and of differences in the extent to which they undergo sorption. Thus univalent ions are possibly only very feebly sorbed, and for this reason the curves obtained are of a similar type to those for solutions of crystalloids, whilst bivalent ions would be more powerfully sorbed. This conception is supported by the results obtained by Białaszewicz [1928] and by his collaborator Zawadzki [1929], who have shown that the percentage of the ions of various electrolytes present in the dispersed phase of the ova of various animals varies greatly according to the valencies of the ions in question. In view however of the circumstance that univalent ions were in every case present in excess in the systems studied by these authors, the above hypothesis requires further experimental confirmation.

If we compare the actions of different salts giving rise to bivalent cations, it will be seen that the action of calcium chloride is very similar to that of magnesium chloride, as is evidenced both by the general similarity of the curves obtained and by the absolute values for reduction of p_H brought about by the presence of equimolar concentrations of both salts. The curves obtained from experiments in which bivalent cations are added to solutions of gelatin, ovalbumin, and peptone are not uniform for each colloid. Thus in the case of albumin a rapid decline in p_H, amounting to 0·21, is observed in concentrations of calcium chloride up to 0·01 M, whilst further addition of this salt up to 0·025 M lowers the p_H by barely 0·03. Gelatin solutions at first exhibit a rise in p_H of 0·30 in 0·005–0·01 M calcium chloride followed by a diminution in p_H amounting in 0·01–0·025 M solutions to about 0·13. The least characteristic curves, and the least resembling adsorption isotherms, are those obtained using peptone solutions, in which case the rapid decline in p_H persists up to 0·02–0·025 M calcium chloride. These differences on the whole support our view as to the fundamental difference in the action of ions on solutions of colloids and of crystalloids, although in all probability they are due partly to differences in the number of buffer groups and in those combining with ions on the surface of the colloidal particles present.

Those experiments performed on systems the p_H of which had been altered by the addition of alkali show that in the vicinity of the isoelectric point the

action of ions is least; as the p_H of the system increases the effect obtained is greater, until at a certain value a maximum effect is observed, after which it again declines. This phenomenon was observed with both gelatin and peptone solutions.

It is of interest that in systems the p_H of which is above the isoelectric [illegible] and in which the colloidal particles should be exceptionally sensitive [illegible]rences in the valency of the ions added, salts giving rise to a univalent [illegible]nd a bivalent anion, *e.g.* sodium sulphate, have a considerably greater act[illegible]han have univalent salts, such as sodium or potassium chloride.

The two following biologically important conclusions may be drawn from the above-described experiments. The first follows from the considerable reduction of p_H brought about by the presence in small concentrations of calcium and magnesium ions. The greatest effects are observed for concentrations within the limits 0–0·005–0·01 M. Since a 0·005 M solution of calcium chloride is equivalent to 55 mg./100 cc. $CaCl_2$, or 19·5 mg./100 cc. Ca, and the calcium content of blood is normally about 10–12 mg./100 cc., it is apparent that small variations from the normal value may exert a certain influence on the p_H of the fluids of the organism. These variations in p_H would depend not on the total calcium content, but on the concentration of this ion in the hydrosphere of the colloidal particles of protein.

The action of calcium ions is, as appeared in the course of further experiments, to a large extent dependent on the presence of sodium and potassium ions, which greatly diminish the magnitude of the effect obtained. Thus 0·01 M calcium chloride reduces the p_H of a 1 % gelatin solution at p_H 5·9 by 0·31, whilst in the presence of 0·150 M potassium chloride the change is only 0·09.

Our second conclusion concerns enzymic reactions, the velocity of which is a function of the p_H of the medium. It follows from Myrbäck's [1926] work on ptyalin that the optimum reaction for this enzyme is at p_H 6·96, whilst at p_H 5·4 and 8·3 the velocity of reaction is only 50 % of that at p_H 6·96. If now the p_H is reduced from 8·3 to 7·8, a difference of 0·5, the observed hydrolysis will rise from 45 to 70 % of that at optimum p_H, *i.e.* the velocity of reaction will be increased by 56 %. The same reduction of p_H below 6·96 will lead to a corresponding inhibition of reaction. The possibility therefore arises that the velocity of enzymic reactions may to a certain extent be regulated by the presence of small concentrations of multivalent ions in systems containing protein.

Whether, under biological conditions, in the presence of high concentrations of univalent ions, this phenomenon plays any important part in the regulation of the velocity of enzymic reactions is questionable. Experiments are however in progress, with a view to the verification of this postulate, and to the determination of its practical importance in vital processes.

SUMMARY.

1. The addition of various mineral salts changes the p_H of solutions of gelatin, ovalbumin and peptone.

2. The depression of p_H is at a maximum on the alkaline side of the isoelectric point.

3. The action of salts is a function not only of their concentration but also of the valency of their ions.

4. The effect produced by unit concentration of salts is greater at very low than at higher concentrations.

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LVIII. THE SELF-SELECTION OF FOOD CONSTITUENTS BY THE RAT.

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Osborne and Mendel [1918] and Mitchell and Mendel [1921] have described experiments in which rats and mice were offered the choice between adequate and inadequate food mixtures. The latter authors summarise their results as follows. "The outcome of this investigation shows in general that in their choice of foods, even between 'synthetic' mixtures which appear to the senses to be essentially alike, rats and mice make selections which are as a rule advantageous for their nutritive condition."

The experiments described below were planned with the aim of ascertaining whether rats would be able to face successfully a similar but probably more complex problem, namely that of choosing for themselves a suitably balanced diet when given access to isolated food components.

In order to simplify the problem as far as possible fats were not made an item of choice for the rats[1]. Similarly a fixed supply of vitamins was given, so the rats were only called upon to choose between three variables—proteins, carbohydrates and minerals.

Experimental.

A litter of six albino rats of Wistar origin, consisting of 3 males and 3 females, was used for the experiment. The animals were weaned at 3 weeks, and kept for 1 week on our stock ration (a slightly modified Steenbock [1923] diet) before being transferred to the experimental diet. Two animals of each sex were placed upon the "free choice" regimen, consisting of caseinogen, sucrose and salt mixture (McCollum's 185) offered in separate cups. The remaining male and female served as controls and were given the same food constituents in the form of a mixture, composed of 75 parts sucrose, 25 parts caseinogen, and 4 parts salt mixture. This diet is known to give good growth when supplemented with cod-liver oil and a suitable concentrate of the B vitamins [Evans and Lepkovsky, 1929].

The caseinogen used in the experiment was prepared in the laboratory from crude commercial "casein" by repeated washing with acidulated water in wooden tubs. It contained 86·5 % protein (N × 6·25) on the dry basis.

Each of the six rats received daily 2 drops of cod-liver oil fed by hand,

[1] Synthetic diets almost free from fat are very well utilised by rats and the amount of fatty substance present in ordinary washed caseinogen and in cod-liver oil is more than sufficient to rule out the possibility of the fat-deficiency disease recently described by Burr and Burr [1929, 1930].

and 0·25 cc. of a watery extract of brewer's yeast [Osborne and Wakeman, 1919] equivalent to 0·5 g. of dry yeast (nitrogen content—8 mg. per dose).

The rats were kept in individual round cages on screens and the nutrients were offered in small glass cups fitted with metal hoods to prevent scattering. This however, especially in the case of the salt mixture and dry caseinogen, could not be entirely avoided, but Petri dishes placed under the cages beneath each food cup permitted a satisfactory separation and recovery of the scattered food constituents and a quantitative estimation of the amounts ingested. The food consumption was recorded daily, except on Sundays. On Saturdays a double portion of the nutrients was weighed out in the food cups, and the food intake for 2 days was measured every Monday. Double rations of the vitamins were also given on Saturdays. The food cups containing the various constituents were always placed in the cages in the same order. They were reversed once during the experiment. Towards the close of the experiment the sugar was replaced by rice starch.

The experiment lasted 10 weeks (69 days). In the course of this time the experimental rats Nos. 116 and 117 died after 54 and 61 days respectively, the first with symptoms of peritonitis, while the cause of death of the second was not ascertained.

Weekly averages of the food consumption of the four experimental rats and a chart comparing the average food intake of the experimental and control rats as well as growth curves of the experimental animals and an average growth curve of the two controls are given in Figs. 1, 2 and 3. All the figures are calculated on a water-free basis, the values for protein refer to 100 % protein (N × 6·25). The non-protein solids in caseinogen were included in the "sugar."

At first glance the figures disclose two salient features: firstly the protein intake of the experimental rats is very low, being in every case less than 10 % (with the exception of the premortal periods of rats Nos. 116 and 117 and of one period for rat No. 115), and secondly their growth increase is small. In fact, if the ability to grow is taken as a measure of the success of the experiment, then it must be said that, on the whole, the rats were unable to select a mixture which would surpass or equal in efficiency the prepared mixture based on conventional standards. On a quantitative or caloric basis the proportions selected by the rats are also far from being economic.

Table I gives the total intake of protein, sugar (and later rice starch), salts and Calories (calculated) for the experimental rats and for the controls.

From these figures the number of Calories consumed per g. increase of weight has been calculated for each rat.

Rat	Calories per g. increase
114	49·6
115	31·1
116	65·4
117	43·2
Average for two controls	22·2

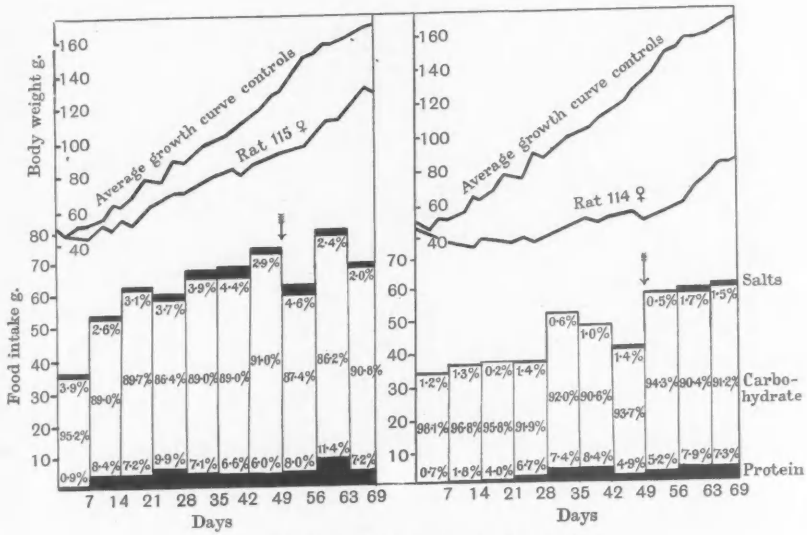

Fig. 1. Weekly food intake of rats 114 and 115.

At ↓ sugar changed to rice starch.

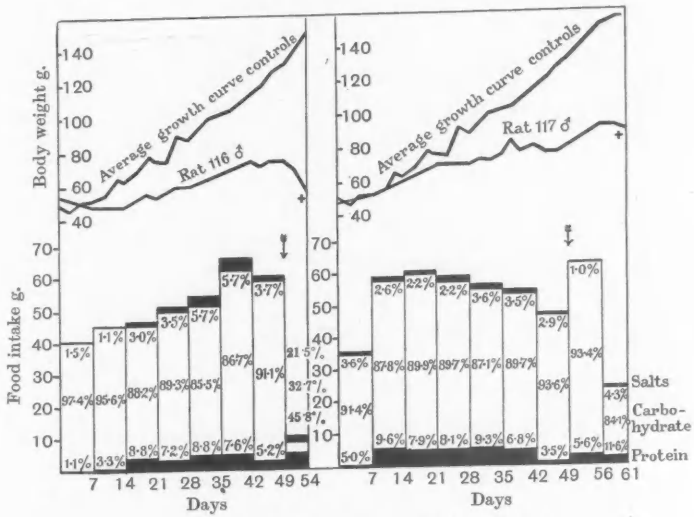

Fig. 2. Weekly food intake of rats 116 and 117.

At ↓ the sugar was changed to rice starch.

When the first seven weekly periods during which all the experimental rats remained alive are taken as a basis, the following average figures are obtained:

	Consumption Calories	Initial weight g.	Weight increase g.	Consumption Calories per g. increase
Control rats	1568	48	82	19·1
Experimental rats	1438	49	26	55·3

A calculation for the last six periods has also been made for rats 114 and 115. It may well be assumed that at this time the rats had become well accustomed to the experimental conditions. Here the figures are more in favour of the experimental rats:

	Consumption: Calories	Initial weight g.	Weight increase g.	Consumption Calories per g. increase
Rat 114	1289	40	43	30
Rat 115	1679	70	59	28·5
Control rats	1891	89	78	24·2

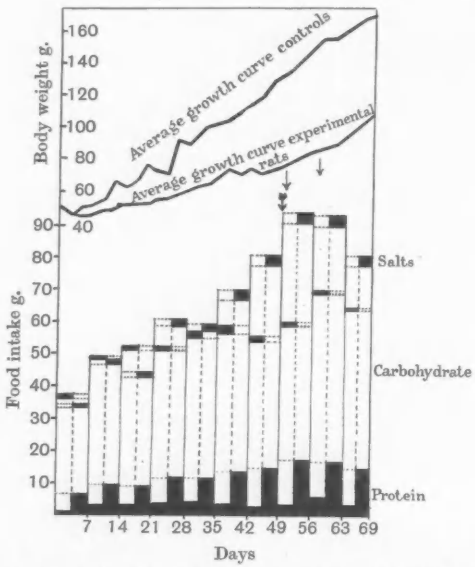

Fig. 3. Comparison of weekly food intake of experimental and control rats.

The left half of each rectangle represents the average food intake of the experimental rats, the right one that of the controls.

The ↓ arrows represent the death of an experimental rat.

The ↯ arrow represents a change from sugar to rice starch.

It must be remembered however that the control animals were heavier than either of the experimental ones and that, according to Hitchcock [1927], "The caloric cost of a gram increase in weight is inversely proportional to the square root of the percentage increase in weight." This of course would

imply that the heavier controls were handicapped when compared with the lighter experimental animals.

It is clearly visible from the curves in Fig. 4 that the animals allowed the choice of food actually consumed more Calories per unit of weight than their

Table I.

Consumption of	Total g.	Daily g.	%	Total Calories	Calories daily
Rat 114. No. of days on experiment: 69; initial weight: 46 g. weight increase: 38 g.					
Protein	26·9	0·39	5·8	110	1·6
Sugar*	432·4	6·27	93·1	1773	25·7
Salts	5·1	0·07	1·1	—	—
Total	464·4	6·73	100·0	1883	27·3
Rat 115. No. of days on experiment: 69; initial weight: 48 g. weight increase: 81 g.					
Protein	48·4	0·70	7·6	198	2·9
Sugar*	565·7	8·20	89·1	2319	33·6
Salts	20·9	0·30	3·3	—	—
Total	635·0	9·20	100·0	2517	36·5
Rat 116†. No. of days on experiment: 49; initial weight: 54 g. weight increase: 22 g.					
Protein	22·7	0·46	6·2	93	1·9
Sugar*	328·1	6·70	90·1	1345	27·4
Salts	13·3	0·27	3·7	—	—
Total	364·1	7·43	100·0	1438	29·3
Rat 117†. No. of days on experiment: 56; initial weight: 47 g. weight increase: 40 g.					
Protein	31·0	0·55	7·2	127	2·3
Sugar*	390·5	6·97	90·2	1601	28·6
Salts	11·3	0·20	2·6	—	—
Total	432·8	7·72	100·0	1728	30·9
AVERAGE FOR CONTROL RATS. No. of days on experiment: 69; initial weight: 48 g. weight increase: 118 g.					
Protein	131·7	1·91	19·8	540	7·8
Sugar*	508·3	7·37	76·6	2084	30·2
Salts	24·1	0·35	3·6	—	—
Total	664·1	9·63	100·0	2624	38·0

* And, later, rice starch.
† The last incomplete premortal period is omitted.

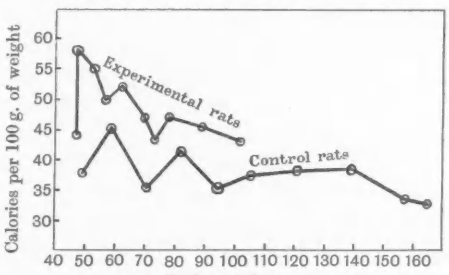

Fig. 4. Consumption of Calories per 100 g. of weight. Weekly averages.

The curve for the experimental rats represents an average for four rats for the first seven weekly periods, for three for the eighth period, and for two for the ninth and tenth periods.

control mates when equal weights are compared, while their growth was at the same time far inferior.

The results are much more favourable for the experimental rats when their weight increments are evaluated on the basis of protein intake. The weight increase per g. of protein ingested can easily be calculated from the figures given in Table I with the following result.

Rat	Weight increase per g. protein
114	1·41
115	1·67
116	0·97
117	1·27

The average protein intake for the four experimental rats for the first 7 weekly periods is 23·5 g. (6·5 % of the total food intake), while the weight increase for the same period amounts to 26 g., a ratio of 1·08. For the last six periods (42 days) the values are:

Rat No.	Protein consumed P (g.)	% of total food intake	Weight increase W (g.)	Ratio W/P
114	21·9	6·7	43	1·96
115	33·1	7·6	59	1·78

It is obviously not possible to compare the experimental rats with the controls in this respect, as the food of the latter contained a rather large percentage of protein, probably well over the requirements for growth at "normal" rate.

On the other hand the values obtained above are nothing else but the biological value of the food protein, caseinogen in this case, calculated according to the method of Osborne, Mendel and Ferry [1919] and may be compared with data found in the literature for caseinogen.

These authors obtained a value for caseinogen of 1·45 at a 9·3 % level in an experiment of 4 weeks' duration and in another experiment a ratio of 1·76 (11 weeks) and 1·77 (4 weeks) at a 9·2 % level. Hoagland and Snider [1926] found a value of 1·98 for caseinogen at a 10·0 % level in a 30 days' experiment. These values refer to levels higher than those encountered in this experiment and, as the maximal values for caseinogen are found, according to Osborne, Mendel and Ferry [1919], at the 12 % level and decrease in both directions, the present figures are at least as good as those reported in previous observations. This finding certainly confirms the obvious supposition that the low level of protein intake was the first limiting factor in the diet freely selected by the experimental rats, but does not supply any additional information as to the reason for this rather surprising choice. It would seem however reasonable to assume, judging from these results, that the poorer growth of the experimental animals was not due to other causes connected with the free selection of their food, such as daily variation of the ratio of various constituents and so on.

As contrasted with the low intake of protein, the voluntary consumption by the "free-choice" rats of the mineral components of the diet in the form

of salt mixture did not differ greatly from the accepted standards, with the exception of rat No. 114. This animal consumed on the average only 1·1 % salts or one-third as much as the remaining three experimental rats. It does not seem however that this low intake of minerals limited the growth of rat No. 114. The protein was here probably the first limiting factor.

Owing to the low voluntary ingestion of protein, sugar formed over 90 % of the diet of the experimental rats (90·6 % as an average for the first 7 weekly periods). It is well known that rats eat sugar very readily and it was thought possible that the factor of taste might have induced the rats to satisfy their caloric requirements almost entirely with a highly palatable carbohydrate to the detriment of the more or less insipid protein. The sugar was therefore replaced by rice starch at the beginning of the 8th weekly period. This change did not however exert the slightest influence on the intake of protein, the only noticeable result being a short temporary drop in the consumption of the carbohydrate.

As already stated the order in which the food cups were placed in the cages of the experimental rats was changed once in the course of the experiment. This had no noticeable effect on the intake of the various food constituents, even on the day of the change.

Discussion.

Perhaps the most noteworthy fact in the above experiment is the voluntary limitation by the experimental rats of their protein intake well below the standards accepted as normal or necessary for the rat. At first sight this result might certainly be interpreted as giving "natural" evidence in favour of low-protein diets and as an objective argument confirming the views of certain students of nutrition. Any such reasoning however would lose its point in face of the poor growth and performance of rats voluntarily subsisting on such diets. The inferiority of the experimental animals as compared with controls consuming a diet richer in protein makes an explanation of their choice highly interesting and desirable but at the same time very difficult. A simple explanation could be offered on the assumption that caseinogen *per se* is distasteful to the rats and that the dislike for this nutrient causes them to limit the intake. It must be remembered however that, when given no choice, the control rats ingested without difficulty adequate amounts of a diet containing 3 times as much caseinogen, and that Osborne and Mendel [1921, 1, 2], Osborne *et al.* [1927], Reader and Drummond [1924–5] and others found quite satisfactory food consumption on diets consisting almost entirely of caseinogen. It is also known that, in search for necessary nutrients, animals subsisting on deficient or faulty diets will consume substances which they would not touch under normal conditions, and which in all probability are distasteful; for example the coprophagy of rats fed on "synthetic" diets, an almost constant phenomenon in all laboratories, the pica or bone-eating disease of cattle and many other examples of depraved appetite. Though a particular

dislike for caseinogen cannot easily be ruled out, it does not seem to afford a sufficient explanation for the rather noteworthy failure of the experimental rats.

Mitchell and Mendel [1921], when offering rats the choice between two diets, one consisting of ground whole corn, with a protein (N × 6·25) content of 9·5 %, the other of meat meal, prepared "by thoroughly drying lean round-steak and then grinding to a meal" and containing 79 % protein (N × 6·25), while a salt mixture was freely accessible in both cases, observed that "contrary to what might have been expected the younger animals selected a smaller proportion of protein in their diets than did the older ones. On the other hand none of the rats chose an excessively 'high-protein' diet although an abundance of the meat meal was always available."

One of the younger animals (rat 21) referred to by Mitchell and Mendel consumed a diet containing only 12 % of Calories in the form of protein; in other words it hardly touched the meat meal. The other (rat 22) consumed 19 % of protein Calories. Both these values are well above the levels ingested by the rats in the present experiment, and, moreover, the rats of Mitchell and Mendel achieved much better growth, rat 21 gaining approximately 85 g. and rat 22 approximately 110 g. in 8 weeks. It must be remembered however that the lowest possible protein intake was for these rats higher than the level voluntarily selected by the animals in the present experiment. Whether the results obtained by Mitchell and Mendel give a truthful picture of the protein requirements of young growing rats or whether they were to any degree complicated or caused by an unequal distribution of vitamins, especially vitamin B, in the two natural foods employed by these authors remains to be answered. Taken as they are they nevertheless point in the same direction as the rather extreme results set forth in this paper.

When the present experiments were planned it was thought that their outcome might throw some light on the much debated question of the optimal level of protein intake and also on its variation in relation to growth (change in nutritive ratio). It seems however that any definite conclusions regarding the "natural" or "instinctive" (whatever these words may imply) protein requirements of young growing rats must be withheld until further experiments with different suitable proteins are instituted.

It has been taken for granted that free choice of regimen is a reliable guide for animals living under normal conditions. It certainly is well worth while to ascertain whether nature is not at a loss in conditions so highly artificial as described in the present paper.

Summary.

1. Young growing rats, offered sucrose (later changed to rice starch), caseinogen and a salt mixture in separate dishes and allowed to make their own choice of these food constituents, consumed on an average only 6·5 % of protein.

2. Changing the sucrose to rice starch did not affect the intake of protein.

3. Two of the experimental rats died in the course of the experiment.

4. Litter mate controls which were given a ready-mixed diet consisting of the same ingredients and containing about 20 % of protein made much greater gains in weight.

5. All rats received equal vitamin supplies, fed separately by hand.

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LIX. A STUDY OF THE NITROGEN BALANCE IN VITAMIN B_2 DEFICIENCY IN THE RAT.

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It was reported in a previous communication [Kon, 1929] that rats kept on a diet deficient in vitamin B_2 show an increased carbon/nitrogen ratio in the urine as compared with individual controls receiving equal amounts of a diet considered to be complete. No such increase was noted in rats consuming a ration deficient in vitamin B_1.

The present paper deals with a quantitative study by the individual control method of the nitrogen metabolism of rats subsisting on diets low in the heat-stable factor (or factors) of yeast.

Experimental.

A litter of six rats of Wistar stock, bred in the laboratory on a slightly modified stock diet of Steenbock [1923] was used for the experiment. They were weaned at 21 days and, after a week on the stock diet, were run out for 17 days on our basal vitamin B-deficient diet 60 (identical with diet 540 of Evans [Kon, 1929]) and consisting of caseinogen (extracted with acidulated water) 25 parts, commercial cane-sugar 75 parts, and Steenbock's salt mixture No. 40, 4 parts. At the end of this time the litter was divided into three pairs, one member of each pair acting as an individual control to its mate. This method has been more amply described in earlier papers by Kon and Drummond [1927] and Kon [1929]. The animals were placed separately in modified Mitchell's cages [Kon, 1928] and offered the following diet: caseinogen (extracted with acidulated water) 10 parts, rice starch 70 parts, butter-fat 15 parts, and salts (Steenbock's mixture No. 40) 5 parts. The diet contained 1·5 % N and 10 % moisture. A quantity of it sufficient for the whole experiment was prepared and kept in cold storage at $-2°$. This diet was selected for several reasons. Firstly in order to avoid spilling as completely as possible and to measure the food intake with a sufficient accuracy it was necessary to offer the food mixed with water to a paste. For this reason rice starch had to be used instead of sugar. Secondly it was thought best for the purpose of the experiment to select a diet low in nitrogen, and containing it at a concentration similar to those used in experiments on the biological value of proteins. Should any deviations from the normal path of nitrogen metabolism manifest themselves in the course of vitamin B_2 deficiency, they would be

most easily detected at such a level of nitrogen intake that most of it, under normal conditions, is used for repair of body tissues and for growth and a small percentage only, if any, burnt as such to cover the caloric requirements. The butter improved the texture of the diet and at the same time supplied vitamins A and D.

The experimental rats were given daily 0·4 cc. of an alcoholic extract from rice bran[1] equivalent to 3·2 g. of bran. This extract was shown in a special experiment to be lacking in vitamin B_2 (and possibly in some other factors supplied in autoclaved yeast). The control animals received an addition of 0·3 g. of dried brewer's yeast of tested potency. In order to equalise the intake of nitrogen an amount of purified caseinogen equivalent in nitrogen content to 0·3 g. of the yeast was placed daily in the porcelain dishes containing the rice bran extract for the experimental animals. The food was mixed with a little water to a pasty consistency to avoid scattering.

For the first four days the food was given to all rats *ad lib.* On the fifth day the residues left in the food cups by the experimental rats were dried at 105° to constant weight, the food intake of each experimental rat was measured and an equivalent amount of the diet was weighed out for the corresponding individual control. The dry residue was discarded from the cups of the experimental animals and fresh food was given slightly in excess of the intake, This procedure was from now on repeated daily, so that the controls were always receiving an amount of food equivalent to that eaten by their mates in the course of the preceding 24 hours. There was practically no scattering and the controls always consumed all the food offered. The yeast, rice bran extract and caseinogen were very readily taken.

The collection of urine and faeces was started after one week of quantitative feeding. This, as well as the analysis of the excreta, was carried out exactly as described in an earlier paper [Kon, 1928]. The rats were weighed once a week, the control animals 24 hours after the experimental ones. The actual metabolic experiment involved five separate collection periods and lasted 36 days, while the quantitative feeding was continued for a week longer (and started a week earlier). The first period lasted 7 days, the following ones 6 days each. At the conclusion of every period the rats were weighed and examined. To avoid any danger of nitrogen losses in the course of handling, an interval of 24 hours separated the periods.

Food consumption.

The food was weighed with an accuracy of 0·01 g. The residues left by the experimental rats were dried for 4–5 hours in an electric oven at 105°

[1] Prepared by extracting rice bran (supplied by Messrs Polski Przemysł Ryżowy in Gdynia) for three days with 25 % by weight alcohol, in the proportion of 4 litres to the kg., concentrating the extract *in vacuo* to a thick syrup, adding a sufficient amount of 95 % alcohol to make the concentration 80 % by weight, decanting the fluid, dissolving the residue in a small volume of hot water, bringing the concentration again to 80 % alcohol, combining the extracts and evaporating them *in vacuo* until 1 cc. is equivalent to 8 g. of the original bran.

until a constant weight was attained. The moisture content of the diet was checked at frequent intervals and was found to remain constant. As the food consumption of the control rats was quite quantitative and the scattering by all the animals negligible (while any food scattered could be easily recovered from the filter-paper on the bottom of a cage), it is felt that the daily difference between the food (diet) intake of a control rat and of its experimental mate did not exceed 0·03 g., the variations being obviously in both directions.

In addition to the diet every control rat consumed 0·3 g. of dried brewer's yeast containing 9·1 % water and 8·81 % N, while the experimental animals received 0·19 g. of caseinogen (with the exception of the first 4 days of the first period, when they were given 0·2 g.) of the same water content and 13·8 % N. The experimental animals were given in addition 0·4 cc. daily of the rice bran extract, containing 2 mg. of nitrogen and 0·164 g. of solid matter, mostly of a carbohydrate nature. This means that the experimental rats consumed daily slightly over 0·06 g. (0·064 g.) of solid matter more than did their controls.

General appearance of rats.

The experimental rats displayed progressively symptoms usually associated with a lack of vitamin B_2, namely rough coats, coagulated blood on paws and vibrissae and urine-stained abdominal fur. The eyes were not involved. One rat (No. 154) had a mild stomatitis. In contrast all the controls were quite normal in appearance and had normal sleek fur.

Weight curves of rats.

In every case the control rats made better gains than their experimental mates. Table I gives the weights at the beginning and end of the quantitative feeding period, while average growth curves for both sets are presented in Fig. 1.

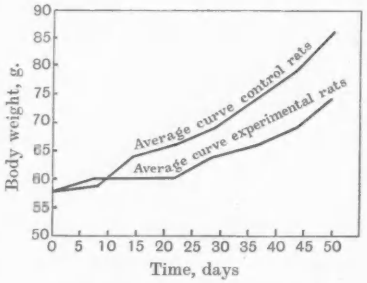

Fig. 1. Average growth curves of experimental and control rats.

During the whole of the experimental period every experimental rat con-

Table I.

Rat no.	Sex	Initial weight g.	Final weight g.	Increase g.	Difference	Remarks
153	♀	56	74	18	+21	Experimental
152	♀	49	88	39		Control to 153
154	♂	65	75	10	+11	Experimental
155	♂	65	86	21		Control to 154
156	♂	56	72	16	+ 7	Experimental
151	♀	63	86	23		Control to 156

Table II. *Nitrogen balance.*

Period	Rat no.	Average weight	Food intake g.	Food intake N mg.	Caseinogen intake g.	Caseinogen intake N mg.	Yeast intake g.	Yeast intake N mg.	Extract intake g.	Extract intake N mg.	Total intake g.	Total intake N mg.	N in urine mg.	N in faeces mg.	Total N excreted mg.	N balance mg.
I	153 E	59	31·45	524	1·24	189	—	—	1·15	14	33·84	727	631	32	663	+ 64
	152 C	59	31·45	524	—	—	1·91	185	—	—	33·36	709	414	65	479	+230
	154 E	63	26·37	439	1·24	189	—	—	1·15	14	28·76	642	589	37	626	+ 16
	155 C	62	26·37	439	—	—	1·91	185	—	—	28·28	624	550	65*	615	+ 9
	156 E	58	31·18	520	1·24	189	—	—	1·15	14	33·57	723	625	40	665	+ 58
	151 C	62	31·18	520	—	—	1·91	185	—	—	33·09	705	521	65	586	+119
II	153 E	59	26·72	445	1·03	157	—	—	0·98	12	28·73	614	565	50	615	− 1
	152 C	63	26·72	445	—	—	1·64	159	—	—	28·36	604	467	58	525	+ 79
	154 E	63	23·61	393	1·03	157	—	—	0·98	12	25·62	562	481	31	512	+ 50
	155 C	65	23·61	393	—	—	1·64	159	—	—	25·25	552	473	33	506	+ 46
	156 E	59	24·42	407	1·03	157	—	—	0·98	12	26·43	576	547	48	595	− 19
	151 C	65	24·42	407	—	—	1·64	159	—	—	26·06	566	465	38	503	+ 63
III	153 E	62	26·78	446	1·03	157	—	—	0·98	12	28·79	615	442	54	496	+119
	152 C	67	26·78	446	—	—	1·64	159	—	—	28·42	605	415	74	489	+116
	154 E	63	21·24	354	1·03	157	—	—	0·98	12	23·25	523	457	28	485	+ 38
	155 C	67	21·24	354	—	—	1·64	159	—	—	22·88	513	400	52	452	+ 61
	156 E	62	23·69	395	1·03	157	—	—	0·98	12	25·70	564	429	45	474	+ 90
	151 C	68	23·69	395	—	—	1·64	159	—	—	25·33	554	421	75	496	+ 58
IV	153 E	67	24·45	407	1·03	157	—	—	0·98	12	26·46	576	468	38	506	+ 70
	152 C	73	24·45	407	—	—	1·64	159	—	—	26·09	566	392	49	441	+125
	154 E	65	22·78	380	1·03	157	—	—	0·98	12	24·79	549	418	35	453	+ 96
	155 C	70	22·78	380	—	—	1·64	159	—	—	24·42	539	415	60	475	+ 64
	156 E	63	23·48	391	1·03	157	—	—	0·98	12	25·49	560	442	38	480	+ 80
	151 C	71	23·48	391	—	—	1·64	159	—	—	25·12	550	422	46	468	+ 82
V	153 E	69	23·08	385	1·03	157	—	—	0·98	12	25·09	554	431	54	485	+ 69
	152 C	79	23·08	385	—	—	1·64	159	—	—	24·72	544	356	46	402	+142
	154 E	68	21·27	354	1·03	157	—	—	0·98	12	23·28	523	343	34	377	+146
	155 C	76	21·27	354	—	—	1·64	159	—	—	22·91	513	343	54	397	+116
	156 E	65	20·30	338	1·03	157	—	—	0·98	12	22·31	507	425	28	453	+ 54
	151 C	76	20·30	338	—	—	1·64	159	—	—	21·94	497	377	62	439	+ 58

* Assumed; the determination was lost.

Table III. *Complete nitrogen balance (36 days).*

Rat no.	Initial weight g.	Final weight g.	Weight increase g.	Food intake g.	Food intake N mg.	Caseinogen intake g.	Caseinogen intake N mg.	Yeast intake g.	Yeast intake N mg.	Extract intake g.	Extract intake N mg.	Total intake g.	Total intake N mg.	Excreted N mg.	Balance N mg.
153 E	58	71	13	152·59	2543	6·21	947	—	—	5·87	72	164·67	3562	3189	+373
152 C	56	81	25	152·59	2543	—	—	9·82	951	—	—	162·41	3494	2696	+799
154 E	64	69	5	135·22	2253	6·21	947	—	—	5·87	72	147·30	3272	2863	+409
155 C	61	78	17	135·22	2253	—	—	9·82	951	—	—	145·04	3204	2857	+347
156 E	58	67	9	142·61	2377	6·21	947	—	—	5·87	72	154·69	3396	3089	+307
151 C	60	78	18	142·61	2377	—	—	9·82	951	—	—	152·43	3328	2889	+439

sumed 3·36 g. solid matter (mostly assimilable carbohydrates) more than his control mate and, nevertheless, made a smaller gain.

Nitrogen balance.

Table II gives the intake and excretion of nitrogen during the five metabolic periods.

It will be recalled that between each metabolic period there was a gap of 24 hours when no collection of urine or faeces was made, the food intake however being recorded in the usual way. In order to calculate the nitrogen balance over the whole duration of the metabolic experiment, it was therefore necessary to include data for the nitrogen excreted in urine and faeces on the days when no actual measurements were made. These figures have been calculated on the probably correct assumption that the nitrogen excretion on the "gap" day would stand in the same ratio to the nitrogen intake as the actual figures obtained for the immediately preceding period. The results are presented in Table III.

An inspection of Tables II and III shows that for the entire period two control rats, Nos. 152 and 151, retained more nitrogen than their experimental mates, while the reverse was true for the control rat No. 155 (which nevertheless gained more in weight than his experimental mate). When the 30 separate measurements carried out in the course of the metabolic experiment are compared in 15 pairs it becomes evident that the control rats were favoured in 9 cases, while the experimental ones retained more nitrogen in 6. It will be noticed that the differences tend to become less marked as the experiment progresses, the values for the second and third pairs of rats running very closely together.

The excretion of faecal nitrogen is almost always higher in the controls (with 2 exceptions), probably due to the consumption of yeast and the production of more bulky faeces [Mitchell, 1924, 1]. It was thought that a clearer picture of the nitrogen metabolism of both groups of rats might be obtained if the biological value of the ingested nitrogen were calculated from the available data by means of the method of Mitchell [1924, 1; Mitchell and Carman, 1926, 2]. Such a calculation necessitates the use of figures for the "metabolic faecal nitrogen" and for the "endogenous urinary nitrogen," usually obtained by measuring the nitrogen excretion of rats during periods of nitrogen-free or of low egg-nitrogen diet. It was not thought safe to include such periods in the work described here, in order not to vitiate the results. It has therefore been necessary to introduce the following assumed factors, based on values obtained previously by Kon [1928] and by Mitchell [1924, 1; Mitchell and Carman, 1926, 2; Mitchell, Beadles and Kruger, 1927], for rats of approximately the same weight as those used in the present experiment. The assumed values are: 2 mg. of metabolic faecal nitrogen per g. of food consumed and 25 mg. of endogenous urinary nitrogen per day per 100 g. of weight. The results of such calculation are presented in Table IV.

Table IV. *"Biological value" of the ingested nitrogen calculated according to Mitchell.*

Period	Rat no.	Average weight	Food intake g.	Food intake N mg.	Faecal N mg.	Body N in faeces mg.	Food N in faeces mg.	Absorbed N mg.	N in urine mg.	Body N in urine mg.	Food N in urine mg.	Food N retained mg.	Biological value
I	153 E	59	33·84	727	32	68	0	727	631	103	528	199	27
	152 C	59	33·36	709	65	67	0	709	414	103	311	398	56
	154 E	63	28·76	642	37	57	0	642	589	110	479	163	25
	155 C	62	28·28	624	65*	56	9	615	550	108	442	173	28
	156 E	58	33·57	723	40	67	0	723	625	101	524	199	27
	151 C	62	33·09	705	65	66	0	705	521	108	413	288	41
II	153 E	59	28·73	614	50	57	0	614	565	88	477	137	22
	152 C	63	28·36	604	58	57	1	603	467	94	373	230	38
	154 E	63	25·62	562	31	51	0	562	481	94	387	175	31
	155 C	65	25·25	552	33	50	0	552	473	97	376	176	32
	156 E	59	26·43	576	48	53	0	576	547	88	459	117	20
	151 C	65	26·06	566	38	52	0	566	465	97	368	198	35
III	153 E	62	28·79	615	54	58	0	615	442	93	349	266	43
	152 C	67	28·42	605	74	57	17	588	415	100	315	273	46
	154 E	63	23·25	523	28	46	0	523	457	94	363	160	31
	155 C	67	22·88	513	52	46	6	507	400	100	300	207	41
	156 E	62	25·70	564	45	51	0	564	429	93	336	228	40
	151 C	68	25·33	554	75	51	24	530	421	102	319	211	40
IV	153 E	67	26·46	576	38	53	0	576	468	100	438	208	36
	152 C	73	26·09	566	49	52	0	566	392	109	283	283	50
	154 E	65	24·79	549	35	50	0	549	418	97	321	228	41
	155 C	70	24·42	539	60	49	11	528	415	105	310	218	41
	156 E	63	25·49	560	38	51	0	560	442	94	348	212	38
	151 C	71	25·12	550	46	50	0	550	422	106	316	234	42
V	153 E	69	25·09	554	54	50	4	550	431	103	328	222	40
	152 C	79	24·72	544	46	49	0	544	356	118	238	306	56
	154 E	68	23·28	523	34	46	0	523	343	102	241	282	54
	155 C	76	22·91	513	54	46	8	505	343	114	229	276	54
	156 E	65	22·31	507	28	45	0	507	425	97	328	179	35
	151 C	76	21·94	497	62	44	18	479	377	114	263	216	45

* Assumed.

Table V gives the average biological values of the ingested nitrogen for all periods for the experimental and control rats. Out of the 15 pairs of

Table V.

Rat no.	Biological value	
153 E	34	
152 C	49	
154 E	36	General average for experimental rats: 34
155 C	39	General average for control rats: 43
156 E	32	
151 C	41	

biological values obtained 3 give equal values for both rats while the remaining 12 are in favour of the control rat, though in 5 cases the difference does not exceed 4, well within the experimental error.

It will be remembered that while the experimental rats derived their

nitrogen almost exclusively from caseinogen, the diet of the control rats contained a considerable proportion (over one-fourth of the total crude protein) of yeast proteins (N × 6·25) in addition to caseinogen. It would be obviously quite simple to use a watery extract from yeast [Osborne and Wakeman, 1919] instead of yeast as a source of water-soluble vitamins for the control rats, but in the light of recent work [Kennedy and Palmer, 1928; Hunt, 1928; Sure, 1928] it seems doubtful whether such extracts contain all the factors originally present in yeast. Preliminary experiments carried out by the author (unpublished work) seemed to confirm this opinion and it was therefore thought safer to avoid the use of extracts.

It might be argued that a supplementary relation exists between caseinogen and yeast nitrogen, yielding a mixture of higher biological value than caseinogen itself and that the differences in weight, retention of nitrogen and biological value of the ingested nitrogen between the experimental rats and the controls might be wholly explained on this basis. The author is not aware of any published work on the biological value of such a combination or of yeast at a 12 % level (*i.e.* at approximately the level of protein used in the present work). He (unpublished experiments) has measured by the method of Osborne, Mendel and Ferry [1919] the biological value of the nitrogen of yeast and has found values of 1·48, 1·31 and 1·36 when fed at a level (N × 6·25) of 8·4, 10·0 and 12·1 % respectively. Osborne, Mendel and Ferry found a maximal value for caseinogen of 2·25 when fed at 12 % level. It does not seem likely that this value would be raised by the addition of yeast. Admittedly this reasoning can only by indirect inference be applied to calculations based on Mitchell's method of evaluating the biological value of proteins. A more direct proof is found in the behaviour of rats Nos. 154 and 155. In this pair the control rat retained less nitrogen than the experimental one, while the calculated biological values of the ingested nitrogen are practically identical for both rats. Nevertheless the control animal gained 21 g. in weight and the experimental only 10 g., a difference equal to 17 % of the initial weight of the rats. It will be also recalled that in the previous communication [Kon, 1929] a distinct difference was reported between the weights of vitamin B_2-deficient and control rats while the former consumed a diet containing a high percentage of caseinogen (25 %), certainly more than necessary for optimal growth if taken in adequate amounts. At such level an increase of the efficiency of caseinogen by the addition of another protein is hardly to be expected.

The biological values calculated by the method of Mitchell for the experimental and control rats are both very markedly below the values found for caseinogen by Mitchell [1924, 2] at a 5 % and by Kon [1928] at an 8 % level, namely 71 and 68 respectively. Mitchell and Hamilton [1929, p. 537] believe that a variable food intake for rats of approximately the same size exerts no effect on the biological values obtained, provided the amount of food consumed is adequate for the maintenance of weight. Mitchell, Beadles and Kruger [1927] noted extremely low figures (zero in several cases) for rats

consuming inadequate amounts of a diet containing pork cracklings as a source of protein, while another group of rats consuming the food better gave a value of 25. The former rats lost weight. In the present experiment all the rats gained weight at a very slow rate, they should consequently, according to Mitchell and Hamilton [1929] give normal biological values (or at least the control rats). There is no doubt that the food intake was subnormal and that the control rats were prevented from growing at a more rapid rate only because their food was limited. It seems possible that at such low levels of food intake a part of the ingested protein may be used for energy purposes while the remainder is used for maintenance and even allows for growth at very slow rate. It is difficult to account otherwise for the very substantial difference between the biological values reported now and found previously for caseinogen.

A calculation of the biological value of the ingested nitrogen according to the method of Osborne, Mendel and Ferry [1919] can be made for the whole period of quantitative feeding covering 52 days. The following values are obtained (Table VI).

Table VI.

Rat no.	No. of days on diet	Consumption of Food	Consumption of Protein (N × 6·25)	Gain in weight g.	Biological value
153 E	52	236·68	32·09	18	0·56
152 C	52	233·32	31·41	39	1·24
154 E	52	212·25	29·54	10	0·34
155 C	52	208·88	28·86	21	0·73
156 E	52	224·02	30·77	16	0·52
151 C	52	220·66	30·10	23	0·76

The values obtained for the experimental rats are markedly lower than those found for the control animals. The difference is much more marked than when the biological value of the ingested protein is calculated according to the method of Mitchell. This result is readily understood in view of the very significant differences in weight gains between the two sets of rats. All the values are much below the figures cited previously for caseinogen fed under usual conditions at approximately the same level. This is due to the insufficient food intake.

Positive nitrogen balances have been obtained in the present paper for all the rats studied. If all of the retained nitrogen was used for building protoplasmic tissue then every gram of nitrogen should cause a gain in weight of 31·25 g. on the assumption that the deposited tissue would contain approximately 20 % protein (N × 6·25).

Table VII gives values calculated on this basis together with the actual gains made by the rats during the metabolic periods.

While for two experimental rats (Nos. 153 and 156) and for one control (No. 152) the calculated values coincide well with the actual gains, the nitrogen retained by rat No. 154 must have been used also for purposes other than the

Table VII.

Rat no.	N retained mg.	Calculated gain in protoplasm g.	Actual gain g.
153 E	373	11·7	13
152 C	799	24·9	25
154 E	409	12·8	5
155 C	347	10·8	17
156 E	307	9·6	9
151 C	439	13·7	18

formation of body protein, and the two control rats, Nos. 155 and 151, would have deposited fat in addition to the formation of the calculated amount of protoplasmic tissue. The only justifiable conclusion is that the gains made by the experimental rats and by the controls were of different composition.

Discussion.

The results here reported were obtained with only a small group of animals (3 pairs) and, though they were rigidly controlled, great caution must be exercised in order not to draw unwarranted conclusions.

Reviewing the evidence on hand the following points seem to deserve special attention.

1. All three control animals made better weight gains than their experimental mates though the former consumed slightly less food. This observation is in agreement with earlier work [Kon, 1929] where, in a similarly controlled experiment, all six controls made better gains than their mates consuming a diet deficient (or low) in vitamin B_2.

2. The nitrogen balance experiments seem rather inconclusive—two experimental rats stored less nitrogen, while one deposited more than the respective controls—and a comparison of 15 pairs of nitrogen estimations shows that the experimental rats were favoured in 6, and the controls in 9, a result easily obtained by chance. The controls however stored on the whole decidedly more nitrogen than the experimental animals. When the biological value of the ingested nitrogen is calculated according to the method of Mitchell a slightly better efficiency is found in the case of the controls, 12 results being in favour of the latter, 3 even, and none in favour of the experimental rats—a result hardly due to chance.

3. There is a strong indication that the gains in weight made by the two sets of rats are of different composition.

4. To this may be added the evidence brought forward in the earlier paper [Kon, 1929] that the carbon/nitrogen ratio is slightly increased in the urine of experimental rats deprived of vitamin B_2, as compared with individual controls.

Is it possible to derive from these observations any conclusions concerning the rôle of vitamin B_2 in metabolic functions? In an effort to answer this question the points enumerated above will be subjected to a more detailed analysis.

(1) The phenomenon described under 1 is not characteristic for vitamin B_2 deficiency. It has been found by other investigators, in well controlled experiments, in the case of various deficiencies and is therefore not pathognomonic of the lack of vitamin B_2. Anderson and Smith [1924] in their study by the individual control method of the effect of acute scurvy on the subsequent nutrition and growth of guinea pigs came to the conclusion that "there are factors resulting in loss of body weight which operate in addition to fasting, and which depend on scurvy itself. In other words, inanition alone does not cause as severe a loss as when accompanied by lack of vitamin C." Mitchell and Carman [1926, 1] have shown by means of the same method that a ration composed mainly of maize, when supplemented with NaCl, induced 50 % faster gains in weight of both rats and chickens than the saltless ration. The authors remark that "the growth data of this experiment afford a striking demonstration of the fact that the utilisation of food energy by growing animals may be greatly impaired by an improper balance among indispensable dietary factors." Similar differences are reported by Mitchell and Beadles [1930] and Beadles, Braman and Mitchell [1930] in the case of diets deficient in cystine and by Jackson [1929] in a deficiency of tryptophan, while Burr and Burr [1930] made observations of the same nature in their study of the fat deficiency disease (individual controls were not employed in this study).

The estimation of the "physiological efficiency" of various proteins, as carried out by the earlier method of Osborne and Mendel [1916], is nothing else than the comparison of food mixtures based on a similar "individual control" method: the rats (all males) were of approximately the same body weight at the beginning of the experiment and were offered daily limited amounts of food "precisely alike in energy content, inorganic and accessory food ingredients, *etc.*" and differing only with regard to the proteins under investigation. The rats made widely divergent weight gains while consuming equal amounts of these rations. On commenting upon these excellent experiments Mitchell [1924, 3] very aptly remarks "these experiments suggest an interesting relation, hitherto uninvestigated as far as the author is aware, between the balance of nutrients in a ration and the utilisation of its energy."

We are here confronting a general phenomenon, namely the growth-limiting influence of the lack or insufficiency of any indispensable food constituent manifesting itself in an impaired utilisation of the food.

For a certain time some vitamins were referred to as "growth-promoting," but there is certainly no doubt that every essential food constituent is growth-promoting in the sense that its absence from the diet will sooner or later cause stoppage of growth. It seems that sufficient evidence was cited above to prove that, as a general rule, one of the ways in which this growth-inhibiting action will come into operation is through an impaired utilisation of the ingested energy, and that in this respect all indispensable nutrients, vitamins, minerals, or amino-acids, manifest a great similarity of action.

A deficiency of an essential ingredient of the diet may or may not have

a depressing influence on the appetite [Beadles, Braman and Mitchell, 1930], at least in the earlier periods of the deficiency. Ultimately probably the general derangement will be sufficiently pronounced to cause in every case a diminished food intake.

The most conspicuous association of an essential food constituent with appetite is certainly to be found in the case of the vitamin B complex and of vitamin B_1. The inanition sets in here so rapidly and is of such severity that no difference in the rate of the loss of weight can be found between "experimental" animals and controls, when the individual control method is used [Kon and Drummond, 1927; Kon, 1929].

(2) Passing now to the second point of interest relating to the study of vitamin B_2 deficiency as presented in this paper, namely to the nitrogen balance experiments, it seems that a less favourable balance would naturally be expected in the case of the experimental rats, having consumed the same amount of, but stored less, energy than the controls, and that therefore the results on the whole agree with the expectation. It was shown by Mitchell and Carman [1926, 1] that more favourable nitrogen balances were found for every one of their control rats and chicks receiving an addition of NaCl, as compared with the experimental ones. The evidence available does not seem to show that vitamin B_2 is any more connected with the metabolism of nitrogen than is sodium chloride.

(3) The question of the composition of the tissues added by the two sets of rats in the present experiment is certainly a very pertinent one. Little more can be said on this point than that the mere estimation of differences in weight does not yield sufficient information, and that in studies of this type, that is to say in the investigation of the relation of any indispensable nutrient to the metabolism of matter and energy, the actual chemical make-up and energy content of the added weight should be estimated as a matter of routine.

(4) Reviewing in turn the value of the finding reported in the previous paper [Kon, 1929], that in the absence of vitamin B_2 there is an increased excretion of carbon compounds as compared with nitrogenous bodies in the urine, it is necessary to ask how significant such an observation is. An alteration of the urinary C/N ratio in scorbutic guinea pigs has been reported by Jarussowa [1928], and Roche [1930] states that the ratio is increased in the terminal stage of vitamin B deficiency. Bickel [1924, 1930] is of the opinion that this symptom is present in disturbances of metabolism of a non-avitaminous character. Laclau and Marenzi [1930] report that the C/N ratio is increased in the urine of rats on a diet poor in cystine as compared with normally fed rats. Shipp and Zilva [1928] have criticised the experiments of Jarussowa and doubt whether the development of scurvy is associated with an increased C/N ratio in the urine. They admit however that their experiments are inconclusive in view of the discordant results obtained (there was a definite increase in the ratio in one of their guinea pigs while no such deviation was observed in the case of the other). While the question can be definitely solved

Page 492, 10th line from bottom:
for vitamin B deficiency *read* vitamin C deficiency

only by means of suitably controlled experiments (with equalised food intake for control and experimental animals), it seems at least doubtful at present whether the alteration of the urinary C/N ratio is characteristic for vitamin B_2 deficiency. It would be best to abstain from far-reaching conclusions, and in particular the author is of the opinion that, while the importance of vitamin B_2 for normal economy is self-evident, there are no sufficient grounds for making it responsible for any particular branch of metabolism.

In conclusion it may be said that while vitamin B_2 is undoubtedly in some way linked with the metabolic processes of the body, it is not at all evident whether this connection is to any extent of a different type from that of the general dependence of normal metabolic exchange upon the availability of any one of the indispensable food ingredients.

SUMMARY.

1. A study by the individual control method of the nitrogen balance in vitamin B_2 deficiency in the rat is reported.

2. The relation of vitamin B_2 to metabolism is discussed.

Note added, April 8th. Still and Koch (1928) have measured, by Mitchell's method, the biological values of diets containing one half of the nitrogen from yeast and one half from caseinogen (total N 2·9 %) and found no supplementary relation between the two proteins.

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LX. THE BIOCHEMISTRY OF DRY-ROT IN WOOD.

II. AN INVESTIGATION OF THE PRODUCTS OF DECAY OF SPRUCE WOOD ROTTED BY *MERULIUS LACHRYMANS*.

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It was shown by us in a previous communication [1929] that, contrary to the usually accepted view, the action of *Merulius lachrymans* (the fungus responsible for dry-rot) on wood was to destroy the major part of the cellulose fraction of the lignocellulose complex and to leave the lignin component unaffected. It was found that the so-called hemicelluloses, which are present in woody tissues, were also resistant to fungal attack, whereas the hexosans, mannan and galactan were completely destroyed.

We have been able to confirm our previous observations as to the general action of the fungus on wood. The present investigation is concerned with the detailed analysis of the nature of the various fractions that can be isolated from rotted wood, together with a comparison of fractions isolated in a similar manner from sound wood.

Experimental.

The material employed was a 5th grade Norwegian spruce wood that had been rotted by *M. lachrymans*, and the various fractions were isolated according to the details given by Dore [1920]. The whole of the wood used (both sound and rotted material) came from the same source, but it was found that the decayed wood was decomposed to a very much greater extent than the sample used in our previous investigation. On this account a quantitative analysis was repeated, and the results are given below.

Compared with our previous sample, the rotted wood used in this investigation shows a considerable decrease in the alkali-soluble fraction (19·10 % compared with 30·0 %). A decrease is also shown in the lignin content (40·90 % compared with 43·00 %) as well as in the value for cellulose (9·88 % compared with 11·90 %), whereas, on the other hand, the alcohol-soluble and water-soluble fractions both show a marked rise over the percentages previously recorded.

Table I.

Treatment	Sound wood % *	Decayed wood % *
Extracted by benzene	1·70	1·48
,, alcohol	1·60	19·80
,, water	2·50	8·00
,, 5 % NaOH in the cold	3·80	19·10
Cellulose	55·00	9·88
Lignin	28·00	40·90
Mannan	7·60	0·00
Galactan	0·10	0·00
Total	100·30	99·16
Ash	0·86	0·61
Natural moisture	10·00	17·30

* Calculated on ash-free material dried at 100°.

The isolation of the various fractions described below was carried out on a large scale. The sample of sound wood was reduced to sawdust and the rotted material was ground to a fine powder in a mortar. The wood (both sound and decayed) was then treated in turn with the reagents described in Table I (*i.e.* benzene, alcohol, water and 5 % NaOH), and the cellulose was isolated after chlorination of the extracted samples and removal of the lignin either with sodium sulphite or with alcohol. Each particular sample was then worked up separately, and the various products were isolated and examined.

It will be convenient to detail the nature of the fractions obtained from the rotted and sound material separately and to discuss the theoretical implications later.

Fractions isolated from rotted wood.

Benzene-soluble fraction. After extraction of the decayed material with benzene and distillation of the solvent, the benzene-soluble fraction was obtained as a viscous, rather mobile, brown mass. This was heated on the water-bath with $N/10$ alcoholic caustic potash for 1 hour and the contents of the flask were then acidified with hydrochloric acid. A heavy brown precipitate separated, which on treatment with ether partially dissolved leaving a brown residue. The residue readily reduced Fehling's solution and formed an oxime. The ethereal extract was distilled under reduced pressure; the residue was taken up with a small amount of $N/10$ sodium hydroxide solution, and the sodium salt of a fatty acid was salted out from the alkaline solution with sodium chloride. Unfortunately the amount of fatty acid obtained was too small to allow of identification. The solution, after the initial precipitation of fatty acid and resin, was distilled under reduced pressure and tested with sulphuric acid and β-naphthylamine for glycerol; a positive reaction was obtained.

The benzene-soluble fraction of rotted wood is therefore composed of a fat or fats and two resins, one of which is soluble and the other insoluble in ether.

Alcohol-soluble fraction. On evaporation of the solvent, there remained a brown, amorphous mass, which partially melted at 60°. It was odourless and

soluble in acetone, glacial acetic acid and sodium hydroxide solution and insoluble in benzene and light petroleum. It was precipitated from glacial acetic acid solution by the addition of water or from sodium hydroxide solution by the addition of hydrochloric acid. It was purified for combustion by dissolving in glacial acetic acid and precipitating with water. The precipitated product was washed and dried *in vacuo*. Analysis indicated the empirical formula, $C_{22}H_{25}O_8$. (Found: C, 63·05 %; H, 6·11 %; $C_{22}H_{25}O_8$ requires C, 63·3 %; H, 5·99 %.) The substance reduced Fehling's solution and the presence of an aldehydic group was confirmed by the preparation of an oxime. Determination of the molecular weight showed that the above formula should be doubled. The substance is therefore not of the nature of the metalignin isolated by Dorée and Barton-Wright [1927], as was previously suggested by us. The substance contained two methoxyl groups (OMe = 9·21 %) and yielded a tetrabenzoyl derivative showing the presence of four hydroxyl groups. (Found: C, 69·0 %; H, 5·6 %; $C_{44}H_{46}O_{12}$ $(O.CO.C_6H_5)_4$ requires C, 69·1 %; H, 5·28 %.) All attempts to separate the material into different fractions failed and from its reactions it appeared to be perfectly homogeneous. Its extended formula can therefore be written $(CH_3O)_2.C_{41}H_{39}O_9(OH)_4.CHO$.

Water-soluble fraction. The water-soluble fraction was acid to litmus and when evaporated to dryness under reduced pressure yielded a dark-brown, viscous mass, which rapidly reduced Fehling's solution. Neither tannins nor organic acids were found to be present, and the nature of the acidity remained undetermined.

The extract was decolorised by boiling with animal charcoal, and methyl alcohol was added to the filtrate, when a copious white precipitate separated. This was found to be entirely composed of glucose (identified by osazone and Bertrand's test). This, with the exception of a trace of acid the nature of which we were unable to determine, was the sole constituent of the water-soluble fraction.

5 % sodium hydroxide-soluble fraction. When the rotted wood, after previous extraction with benzene, alcohol, and water was treated for 36 hours in the cold with a 5 % solution of sodium hydroxide, a dark-brown liquid was obtained. This was filtered off from the residue and together with the first washing was treated with an excess of acetic acid, which produced a flocculent brown precipitate. After standing for 24 hours, the supernatant liquid was poured off, and the precipitate was washed several times with water to free it from acid. It was air-dried for several days and finally dried *in vacuo*. During the course of drying it became very much darker in colour.

The supernatant liquid, which was of a pale straw colour, was dialysed for 8 days to free it from acetic acid and sodium acetate and then evaporated to small bulk under reduced pressure and treated with an equal volume of alcohol. The small amount of dark-brown precipitate was filtered off, washed with alcohol and dried *in vacuo*. The filtrate was evaporated to dryness, when a dark-brown evil-smelling mass was left as a residue.

The alkali-soluble fractions of wood contain the so-called "hemicelluloses," bodies which are composed of hexose and pentose sugars as well as uronic acids of the galacturonic and glycuronic type. A number of investigations have recently appeared on these compounds. O'Dwyer [1926, 1928], for example, was able to isolate two such bodies from beech wood sawdust, to which she gave the names of hemicellulose A and hemicellulose B respectively. Hemicellulose A was insoluble in water, but soluble in dilute solutions of sodium hydroxide, from which it could be precipitated by acids; hemicellulose B was soluble in water, but insoluble in alcohol. The two substances which we have been able to isolate from decayed spruce wood appear to be of the nature of hemicelluloses, but owing to the fact that they showed a number of differences from the products isolated by O'Dwyer, as well as from the corresponding fractions isolated from sound spruce wood, we prefer to name them hemicellulose A_1 and hemicellulose B_1.

Hemicellulose A_1. This was a dark-brown amorphous body, insoluble in water, but slightly soluble in alcohol. It was readily soluble in weak solutions of sodium hydroxide and could be reprecipitated from alkaline solution by the addition of acid. It reduced ammoniacal silver nitrate but not Fehling's solution.

20 g. of the material was hydrolysed with 200 cc. of 3 % sulphuric acid for 6 hours. At the end of this time the residue was filtered off, and the requisite amount of barium hydroxide was added to neutralise the sulphuric acid. The precipitate of barium sulphate was filtered off, and the filtrate was found to be still slightly acid. A further quantity of barium hydroxide was added until the solution was alkaline, and the precipitate was filtered off and preserved. The filtrate was evaporated under reduced pressure to a thin syrup and extracted several times with absolute alcohol and then with 80 % alcohol.

The two alcoholic extracts were tested for mannose as the hydrazone, arabinose as the osazone, xylose as the cadmium xylobromide salt, galactose as mucic acid and glucose as the osazone. The portion soluble in absolute alcohol gave the characteristic crystals of cadmium xylobromide and showed the presence of xylose in the complex. No other sugar was found to be present in this portion. The extract obtained with 80 % alcohol gave galactosazone melting at 189–190°. The presence of galactose was confirmed by the preparation of mucic acid.

The percentage of uronic acid in the complex, estimated by the method of Nanji, Paton and Ling [1925], was 10·73 %. The second barium precipitate mentioned above was treated with the calculated quantity of sulphuric acid to decompose the barium salt of the uronic acid. The barium sulphate was filtered off, and the filtrate was saturated with cinchonine at 100°. On cooling, large crystals of cinchonine sulphate separated out, owing to the presence of a slight excess of sulphuric acid in the mixture; at the same time, a mass of fine needle-shaped crystals was also obtained. The fine needles were centrifuged off, freed from cinchonine sulphate by washing with alcohol and twice

recrystallised from water (M.P. 211–214°). We have been unable to find in the literature a uronic acid the cinchonine salt of which melts at this temperature. Up to the present time, the only uronic acids that have been isolated from hemicelluloses are galacturonic and glycuronic acids. It is possible that we are here dealing with a penturonic acid, that derived from arabinose or xylose. Gymnospermous woods have not been investigated with regard to the nature of their hemicellulose content, and we are at present engaged in making a fuller examination of this point, as well as in preparing pure samples of the uronic acids from xylose and arabinose for purposes of comparison.

When treated with fuming hydrochloric acid (sp. gr. 1·20) hemicellulose A_1 after 20 hours yielded a lignin-like body having OMe 12·4 %. The original sample before acid treatment had OMe 6·68 %.

The nature and amounts of the products obtained from hemicellulose A_1 are shown in Table II. An attempt was made to determine the nature of the

Table II.

	%
Xylose	1·17
Uronic acid (nature unknown)	10·73
OMe of hemicellulose A_1 before treatment with HCl	6·68
OMe of residue after treatment with HCl (*sp. gr.* 1·2)	12·40
OMe of residue calculated on original material ...	6·15
OMe hydrolysed by HCl	0·53
Residue insoluble in HCl	49·64

linkages between the component parts of the molecule. Portions of 5 g. of the hemicellulose A_1 were hydrolysed with 3 % sulphuric acid for 1, 3, 5 and 10 hours respectively and the methoxyl content, furfuraldehyde and carbon dioxide values were determined. The figures, calculated as a percentage of the original material, are given in Table III. It is clear that the linkages between

Table III. *Effect of acid hydrolysis for varying periods on hemicellulose* A_1.

Fraction	Original material %	1 hour %	3 hours %	5 hours %	10 hours %
Xylose	1·17	0·00	0·00	0·00	0·00
Uronic acid	10·73	4·10	4·40	4·40	4·89
Methoxyl	6·68	6·52	6·45	6·30	6·00

the component parts of the complex differ from one another. Part of the methoxyl is hydrolysable by dilute acid, and the ease of hydrolysis suggests that the linkage is of an ester type, while the remainder of the methoxyl is attached to the portion insoluble in fuming hydrochloric acid, *i.e.* the lignin-like fraction, and the linkage here is of a more resistant nature, probably of an ether type.

The uronic anhydride content exhibited a peculiar feature, namely, a large initial decrease (7 %) after hydrolysis for one hour, but showed an increase during the remaining period. This result again suggests that there is more

than one type of linkage present in the complex. During the first hour, a weak linkage is destroyed, but during the remaining period over which hydrolysis is continued, a more resistant linkage is attacked, and one which prevents complete decarboxylation from taking place when uronic acids are estimated by the method of Nanji, Paton and Ling. The mild acid hydrolysis breaks this linkage slowly, and the yield of carbon dioxide recorded on decarboxylation represents the sum of the yields of gas from the acid set free by the mild acid hydrolysis and that set free by 12 % hydrochloric acid in the course of estimation. In order to nullify the effect of the latter hydrolysis, the time during which the estimations were carried out was kept constant. In this way the effect of the mild hydrolysis became apparent.

The fraction of rotted wood soluble in sodium hydroxide solution can therefore be divided into two portions, a part insoluble in fuming hydrochloric acid, which contains methoxyl groupings, but yields no carbon dioxide or furfuraldehyde when treated with hydrochloric acid, and may well be a body of lignin-like nature; and a second part composed of a mixture of pentose (xylose) hexose (galactose) and an unknown uronic acid. These constituents may be removed separately from the complex, together with a certain amount of methoxyl residues. The linkages between the xylose, galactose and uronic acid have not been completely defined by the methods employed, but it is evident that the pentose is more loosely combined in the complex than galactose and that the uronic acid is linked in such a manner that it is shielded from decarboxylation. This might be achieved by esterification or by the linkage of one of the other components of the complex to one of the carbon atoms in the uronic acid molecule so that a protective action is exercised over the carboxyl group. The possibility of esterification preventing decarboxylation is remote. Until we have carried out further work on the point, the first alternative will have to stand.

Hemicellulose B_1. Hemicellulose B_1, isolated in the manner described above, was a dark-brown powder, readily soluble in water. Unfortunately the amount available was too small to allow of any extensive observations being made on its properties. It contained 7·12 % of pentose; the methoxyl content was 3·19 % and the uronic acid value 7·68 %. When treated with hydrochloric acid (sp. gr. 1·2) for 24 hours it gave an insoluble residue of 31·88 %. It thus appears that here, as in the case of hemicellulose A_1, the true hemicellulose portion of the molecule is linked with a lignin-like fraction.

Cellulose fraction. The cellulose was isolated from decayed wood by chlorination after previous extraction in the cold with 5 % sodium hydroxide and subsequent removal of the chlorolignin by repeated extraction with boiling alcohol. It could also be isolated by boiling the chlorinated product with sodium sulphite. Both methods gave similar results. The residue, after extraction of the lignin with alcohol, was air-dried, when it was obtained as a hard dry mass, which could be reduced to a fine powder by grinding in a mill. It showed little fibrous structure. Small portions of the material were treated

with calcium hypochlorite, but in no case was any bleaching action observed. Uronic anhydride amounted to 3·23 % and pentose to 0·39 %. The physical nature of the material, together with the increased uronic acid value compared with cellulose isolated from normal wood (see below), suggested that the cellulose had suffered oxidation under fungal action; tests for oxycellulose were therefore carried out. The material rapidly reduced Fehling's solution and showed a marked affinity for basic dyes. With α-naphthol and sulphuric acid it developed a violet colour and with thymol and sulphuric acid a rose tint. Subjection to the Cannizzaro reaction abolished the reducing properties. The residue after treatment with strong sodium hydroxide solution was filtered and washed free from alkali and then tested with Fehling's solution; no reduction occurred. The filtrate, when acidified with hydrochloric acid, yielded a white precipitate which also failed to reduce Fehling's solution. The filtrate was neutralised and tested with Fehling's solution, but showed no reducing properties. Presumably an aldehydic grouping present in the cellulose of the decayed wood had suffered simultaneous oxidation and reduction under the influence of the Cannizzaro reaction, giving rise to an insoluble alcohol and the sodium salt of an insoluble acid. These tests indicate that part at least of the cellulose contained in rotted wood consists of oxycellulose. Treatment with 17·5 % sodium hydroxide solution according to the method of Schorger gave a yield of 22·9 % of α-cellulose. No attempt was made to estimate either β- or γ-cellulose.

Lignin fraction. The alcoholic solution obtained from the extraction of chlorolignin was distilled under reduced pressure until the chlorolignin began to be deposited as a hard brown mass on the bottom of the flask. This was removed, ground to a fine powder and washed with a small amount of water until free from sodium chloride (which remained from an earlier brine treatment of the chlorinated wood).

The chlorolignin thus isolated could be separated into two fractions: one soluble in glacial acetic acid and one soluble in sodium hydroxide solution. The chlorolignin was first treated with glacial acetic acid in the cold or occasionally with gentle warming, until after repeated extraction no more would pass into solution. The residue was washed free from acid and treated with a 1 % solution of sodium hydroxide. The acid fraction was isolated by pouring into a large volume of water, when a flocculent brown precipitate settled out. This was filtered off, washed, and dried *in vacuo*. The portion soluble in dilute sodium hydroxide solution was precipitated by the addition of dilute hydrochloric acid, washed free from acid and dried *in vacuo*.

Glacial acetic acid fraction. Further purification for combustion was effected by re-dissolving in glacial acetic acid and again precipitating with water. (Found: C, 47·6, 47·4 %; H, 3·72, 3·6 %; Cl, 19·22, 19·07 %.) No formula can be constructed from these figures and the acetic acid-soluble fraction is evidently a mixture. Further evidence that a mixture had been obtained can be seen from the figures found for the combustion of the benzoyl derivative,

in which the percentages for hydrogen are higher than for the original product. (C, 54·64, 54·22 %; H, 4·48, 4·33 %; Cl, 11·91, 11·61 %.)

Sodium hydroxide-soluble fraction. This also proved to be a mixture and the figures found for carbon on combustion showed an even greater variation than those obtained for the acetic acid-soluble fractions. (C, 43·05, 43·15, 48·1, 48·9 %; H, 4·09, 3·85, 3·68, 4·07 %; Cl, 15·87, 15·30 %.)

Fractions isolated from sound spruce wood.

For the purpose of comparison, fractions were now isolated from sound spruce wood in exactly the same manner as from the decayed material. The wood was reduced to sawdust by running the end of a block against a fast-running circular saw. In this way 1400 g. of material were prepared. This sawdust was reduced to wood flour of the same grade as the rotted sample by grinding in a mill and was extracted successively with benzene, alcohol, water and 5 % sodium hydroxide solution in the cold. The cellulose was isolated by chlorination of the wood after alkaline extraction followed by boiling with alcohol or sodium sulphite.

Benzene-soluble fraction. On evaporation of the solvent, a practically colourless, odourless, slightly viscous resinous mass remained. This was boiled for one hour with $N/10$ alcoholic caustic potash; the contents of the flask were then acidified with hydrochloric acid and the precipitate of fatty acids and resins filtered off. On extraction with ether, the resins entirely passed into solution, together with a portion of the fatty acids. The ethereal extract was evaporated to small bulk, and the residue was dissolved in a small amount of sodium hydroxide solution, from which the fatty acids were salted out, leaving the resins in solution. The filtrate, after initial acidification with hydrochloric acid, was distilled under reduced pressure and tested for glycerol with β-naphthylamine and sulphuric acid: a positive reaction was obtained.

The benzene-soluble fraction isolated from sound wood, therefore, like that of the rotted material, is composed of fats and resins.

Alcohol-soluble fraction. After removal of the alcohol by distillation under reduced pressure, a small amount of a dark-brown, oily liquid remained. This was found to be insoluble in glacial acetic acid and was too small in amount for identification.

Water-soluble fraction. On evaporation of the aqueous extract under reduced pressure, a dark-brown, slightly viscous mass remained. This reduced Fehling's solution and tests with methylene blue showed it to be composed of tannins and reducing sugars in small amount.

Sodium hydroxide-soluble fraction. The residue of the sound wood after extraction with benzene, alcohol and water was allowed to stand in a 5 % solution of sodium hydroxide for 36 hours in the cold. At the end of this period the residue was filtered off yielding a golden-brown solution. The first washings from the residue were added to the filtrate and the whole was acidified with acetic acid. A small amount of a light-coloured precipitate

separated out. The supernatant liquid was removed and the precipitate was washed free from acid and dried *in vacuo*. Unlike the corresponding precipitate from decayed wood, it underwent no darkening in colour on drying. The filtrate, after acidification, was dialysed for 8 days to remove excess of acetic acid and sodium acetate and was then evaporated to small bulk under reduced pressure. When alcohol was added, a slight precipitate was formed, but this was too small in amount to collect.

The first precipitate which separated on acidification of the alkaline extract presumably corresponds with the hemicellulose A of O'Dwyer and the second, which formed on the addition of alcohol, to her hemicellulose B. In any case her nomenclature will be followed here for the sake of convenience and to save confusion.

Hemicellulose A. The fawn-coloured precipitate formed on the addition of acid to the alkaline extract of sound wood was treated with fuming hydrochloric acid (sp. gr. 1·2) for 24 hours and left a residue of 47·7 %, which had OMe 12·72 %. The original material had OMe 7·87 %, and contained 3·61 % of uronic anhydride and 5·94 % of pentose.

12 g. of the material were hydrolysed with 3 % sulphuric acid for 5 hours at 100°. At the end of this time, the residue was filtered off, washed and dried and amounted to 55·9 % of the original material. The filtrate was neutralised with barium hydroxide in exactly the same way as for the fraction isolated from decayed wood, and the precipitate of barium sulphate was removed and preserved. The residue of the neutralised filtrate after evaporation was extracted first with absolute alcohol and then with 80 % alcohol, and the two extracts were tested for mannose, galactose, arabinose and xylose. The absolute alcoholic extract was found to contain xylose and the 80 % alcoholic extract galactose, while the barium sulphate precipitate contained the unknown uronic acid described above for hemicellulose A_1. The constitution of hemicellulose A is summarised in Table IV. Owing to the small amount of

Table IV.

	%
Xylose	5·90
Uronic acid	3·61
OMe	7·89
Residue after treatment with HCl (*sp. gr.* 1·2) ...	47·70
OMe in residue after treatment with HCl	12·72
OMe calculated on original material	6·07
OMe readily hydrolysed by HCl $\left(7{\cdot}89 - \frac{12{\cdot}72 \times 47{\cdot}7}{100}\right)$	1·82

material available it was impossible to carry out a series of hydrolyses over varying periods of time, as in the case of hemicellulose A_1. We have, on this account, no evidence as to the nature of the linkages between the component parts of the complex.

Cellulose. The cellulose was isolated after alkaline extraction of the sound wood and chlorination, by dissolving out the chlorolignin either with alcohol

or with alkaline sodium sulphite. The percentages obtained were the same in both cases; 55·0 % as compared with 9·88 % in rotted wood.

The cellulose was a white fibrous substance, which did not reduce Fehling's solution and showed none of the properties of oxycellulose. It yielded 80·65 % of α-cellulose and 8·97 % of pentosan, calculated from the furfuraldehyde precipitated as phloroglucide, after making allowance for the uronic acid content. The percentage of uronic acid was 0·95 %.

Lignin fraction. The chlorolignin was isolated from the sound wood in the same manner as described above for decayed material. Separation into two fractions was again effected by means of glacial acetic acid and 1 % sodium hydroxide solution. On combustion the samples gave the following values:

Glacial acetic acid fraction: C, 47·89, 48·77, 52·9, 42·1, 43·1 %; H, 4·54, 4·94, 4·79, 4·66, 5·42 %; Cl, 18·77 %; OMe, 4·7 %.

Sodium hydroxide-soluble fraction: C, 49·53, 53·14, 50·77 %; H, 4·03, 4·87, 4·77 %; Cl, 12·93, 12·83 %.

From the wide variations in these figures, it is clear that, as in the case of the chlorinated products isolated from decayed wood, we are dealing with mixtures and not homogeneous products. The main difference lies in the fact that the samples isolated from fungus-infected wood possess a higher percentage of oxygen than the corresponding samples from sound material.

Discussion.

It is necessary to emphasise the fact that throughout the course of this investigation only completely decomposed material was employed. We are, on this account, unable to advance any views as to the exact course of fungal metabolism at each stage in the progress of decay. It is clear, however, that the presence of the fungus in the wood produces far-reaching and profound changes in the wood components, especially in the two most important fractions of the lignocellulose complex, namely, the cellulose and lignin.

The fact that the cellulose has fallen by 81 % in the rotted material and that the residue remaining is much altered in nature, indicates that the chief action of the mycelium is exercised on this particular component of the woody tissues. Two reactions appear to have progressed simultaneously here: a hydrolysis of the cellulose to glucose and an oxidation to oxycellulose. Presumably the comparatively large amount of glucose located in the water-soluble fraction of the rotted wood was originally derived from the cellulose and was left as an unconsumed residue by the fungal mycelium in its passage through the wood. In any case, it is the α-cellulose fraction of the total cellulose of the wood that is chiefly affected by the presence of the fungus, as there is a fall from 47·6 % to 2·26 % in α-cellulose, calculated on the total wood.

It was shown by us in our previous communication that the hemicellulose fractions of the wood were apparently unaffected by fungal infection; although at the time we were unable to account for the increased solubility of decayed

wood in sodium hydroxide solution. It is very unfortunate in this connection that different workers have defined the term hemicellulose in different ways. Candlin and Schryver [1928], for example, have introduced the term "polyuronide" to include substances containing linked uronic acids and sugars, whether the latter be pentoses or hexoses. Such a definition includes pectins as well as hemicelluloses of the type described by O'Dwyer and gums of the gum arabic type recently described by Norman [1929]. More recently, Norris and Preece [1930] have agreed with the classification put forward by Candlin and Schryver and have further suggested that the term hemicellulose should be restricted to bodies which give no uronic acids but only sugars on hydrolysis. It appears to us superfluous to introduce the term polyuronide in this connection, since it brings into the same class such diverse bodies as the pectins and hemicelluloses isolated by O'Dwyer, while the terms hexosan and pentosan still appear to be quite adequate to describe compounds only containing hexoses or pentoses, and some synthetic name may be derived for products consisting of mixtures of these two classes of sugars. The term hemicellulose is used here for products soluble in dilute sodium hydroxide solution, but insoluble in sodium carbonate solution, which on hydrolysis yield hexose and pentose sugars as well as uronic acids. Using such a definition, it will be seen that the hemicellulose products of wood are practically unaffected by *M. lachrymans*. In both sound and rotted wood, the same products are obtained on hydrolysis, namely, xylose, galactose and an unknown uronic acid and in much the same proportions. A residue insoluble in fuming hydrochloric acid is also found in both cases and it is curious that no mention is made by either O'Dwyer or Norman that a residue remains when this material is hydrolysed by weak acid. The fact that a residue of nearly 50 % remains after treatment with fuming hydrochloric acid for 24 hours considerably invalidates the Willstätter and Zechmeister [1913] method of estimating lignin in woody tissues.

It is peculiar that the fungus fails to remove the hemicellulose fraction of the wood, since the hemicelluloses contain both hexose and pentose sugar in their composition. On the other hand, *M. lachrymans* is markedly sensitive to the action of acids and even such weak acids as tannic and gallic acid inhibit its growth and produce a toxic effect. Since uronic acids are produced by the hydrolysis of hemicelluloses, it may well be on this account that the fungus fails to attack the hemicellulose fraction.

Campbell and Booth [1929] have investigated the action of *Trametes serialis* on Sitka spruce and like other investigators have observed an increase in the solubility of the rotted material in dilute solutions of caustic alkalis, as well as a decrease in cellulose content. From the sum of the percentages of the fractions in the rotted wood, they have put forward the suggestion that a substance is formed, which, while insoluble in water, is not estimated as either cellulose, lignin or pentosan and is possibly of the nature of a carbohydrate degradation product. From a comparison of the effect of the fungus

on sawdust with the action of an aqueous acid hydrolysis at 100° they have formulated the view that, in the earlier stages at least, the effect of infection of woody tissues by a "brown-rot" type of fungus is to be regarded as strictly comparable with aqueous acid hydrolysis and consider that support for this suggestion is to be found in the acid nature of rotted wood. In the absence of any experimental work on the nature of the alkali-soluble fraction of rotted material, it appears somewhat premature to advance any theory to account for the effects of fungi of the "brown-rot" type. In the later stages of decay, our results indicate that oxidation plays a very much more prominent part in the process than anything akin to an aqueous acid hydrolysis.

Although the total lignin content of wood is unaffected by *M. lachrymans*, nevertheless, the fungus produces a number of changes in this component of the lignocellulose complex. One curious feature of the problem is the occurrence of a definite compound of composition $C_{44}H_{50}O_{16}$ in the alcohol-soluble fraction of decayed wood. This substance shows the reactions ordinarily associated with an "alkali" lignin and very closely approximates in composition to the lignin isolated by Powell and Whittaker [1924, 1925] from flax by treatment of the fibres with 8–12 % solutions of sodium hydroxide under pressure for several hours. We had hoped that this product would have been a substance of simpler composition and possibly of the nature of "metalignin" isolated by Dorée and Barton-Wright [1927], which possessed the molecular composition, $C_{20}H_{20}O_6$.

It is difficult to account for the presence of this lignin in the alcohol-soluble fraction. It was the only homogeneous lignin product we were able to isolate throughout this investigation. The majority of investigators of the chemistry of lignin have assumed that the lignin fraction of the lignocellulose complex is a homogeneous body and numerous formulae have been put forward from time to time. From the results of our present work, this homogeneity does not appear to exist. The chlorolignins that were isolated, for example, from both sound and decayed wood were all mixtures. It is possible that lignin, using the name generically, is composed of closely related isomerides, perhaps differing only in methoxyl content, and, since they are colloidal in nature, it may be on this account that apparent homogeneity has been found, where in fact none existed. But whether lignin be a single substance or a mixture, it is highly susceptible to oxidation and is able to take up large amounts of oxygen [*cf.* Dorée and Hall, 1924; Dorée and Barton-Wright, 1929]. Apparently a similar result is brought about by the action of a "brown-rot" fungus on wood. The chlorolignins isolated from decayed wood all possessed a higher oxygen content than similar bodies isolated from sound material.

The action of *M. lachrymans* on wood is therefore to consume the hexosans, mannan and galactan, and then to attack the cellulose fraction of the complex. The latter is converted to glucose, the α-cellulose being primarily affected, while a small residue is left as oxycellulose. The hemicelluloses are not destroyed and the lignin fractions, though not removed, suffer oxidation.

SUMMARY.

1. A detailed investigation of the action of *Merulius lachrymans* (the fungus of dry-rot in wood) has been made on spruce wood. The action of the fungus is first to remove the easily hydrolysable fractions, mannan and galactan, and then to attack the cellulose. The latter is converted into glucose and consumed as such. At the same time a small residue of oxycellulose is left.

2. A homogeneous lignin body was extracted from the rotted wood with alcohol, which showed the molecular composition $C_{44}H_{50}O_{16}$ and possessed the extended formula $(OCH_3)_2.C_{41}H_{33}O_9(OH)_4.CHO$. From its reactions it appeared to be similar to the lignins which have been isolated from woody tissues by the action of caustic alkalis under pressure.

3. The hemicellulose fractions of spruce wood are unaffected by the fungus. An unknown uronic acid was isolated from the hemicellulose A fraction. The suggestion is put forward that this is a penturonic acid.

4. The total lignin content of the wood is practically unaltered, but marked oxidation has taken place, *i.e.* there is little or no delignification of the wood.

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LXI. THE GLYCERIDE STRUCTURE OF BUTTER-FATS.

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The glyceride structures of five butter-fats from cows fed under definite conditions have been investigated by isolation of sufficient of their fully-saturated glycerides to permit in each case of a detailed analysis of the fatty acids present in the latter by the ester-fractionation process. The component fatty acids of the whole butter-fats had already been determined [Hilditch and Sleightholme, 1930]. Combination of the analytical results for the whole fats with those for the fully-saturated glyceride portions furnishes much information as to the distribution of saturated and unsaturated acids among the glycerol molecules, and also throws some light on certain aspects of the distribution of the individual saturated acids. The objects of this communication are (i) to summarise the experimental data on the examination of the fully-saturated glycerides present in these five butter-fats and (ii) to discuss the general features of the glyceride structure of butter-fats revealed by these figures in conjunction with those formerly obtained for two New Zealand butter-fats [Hilditch and Jones, 1929] and, more recently, for an Indian cow ghee [Bhattacharya and Hilditch, 1931].

The various specimens will be referred to throughout this paper as follows.

I. Shinfield, Berkshire; October 1928, cows partly on pasture, partly on winter diet.

II. Shinfield, Berkshire; March 1929, cows on winter diet with added coconut cake ration.

III. Shinfield, Berkshire; March 1929, cows on winter diet with added soya bean cake ration.

IV. Shinfield, Berkshire; May 1929, cows on early summer pasture.

V. Palmerston North, New Zealand; December 1928, cows on early summer pasture.

A and B. New Zealand butter, market samples [Hilditch and Jones, 1929].

CG. Indian cow ghee from pasture-fed cows [Bhattacharya and Hilditch, 1931].

The butters I–IV were collected from members of the same herd of ten Shorthorn cows at the National Institute for Research in Dairying and V was

supplied by Dr F. H. McDowall from the Dairy Research Institute at Palmerston North; details of the feeding of the cows in question will be found in our former paper [1930, pp. 1099, 1100], and the general analytical characteristics of these five butter-fats and the data for the component fatty acids present in the whole fats have also been recorded [1930, pp. 1100, 1105, 1106].

Fully-saturated glyceride content of butter-fats I–V.

Determinations of fully-saturated glyceride content were carried out by the method which has been fully described in several recent papers [*cf.* Hilditch and Jones, 1929], the fat, in quantities of 100 g., being dissolved in acetone (1 litre) and oxidised by gradual addition of powdered potassium permanganate (400 g.) to the gently boiling solution. The only modification which has been made in the procedure as formerly described is that it has been found useful to have suspended in the solution clean dry silver sand (about 300 g.), which lessens the accumulation of a hard cake of mixed fat, oxidation products and manganese oxides, and facilitates its removal for subsequent reduction of the oxides of manganese by means of sodium bisulphite and dilute sulphuric acid. After separating as much of the acidic products of oxidation as possible by washing the ethereal solution with dilute aqueous ammonia, the crude fully-saturated glycerides were recovered from the washed ethereal solution and in all cases possessed a negligible iodine absorption (less than 0·2 %). Usually 500–600 g. of each fat was oxidised, 100 g. at a time, in order to provide an adequate quantity of purified fully-saturated glycerides for the detailed analysis of the component fatty acids present.

The crude fully-saturated glycerides thus obtained were further purified from any remaining acidic products of oxidation by adding a boiling saturated solution of potassium carbonate to the melted crude glycerides in an evaporating basin, with vigorous stirring, until a stiff paste was formed; this was immediately boiled with a large quantity of water until clarification was complete, allowed to settle, and the aqueous solution siphoned away from the upper layers of liquid fat and emulsion. This washing process was repeated until no emulsified paste was left, when a further washing with boiling aqueous potassium carbonate was given and the residual fat finally washed free from soap and alkaline carbonate by the above method. The alkaline and aqueous washings were collected together and thoroughly shaken with ether, and in this way two weighed fractions of fully-saturated glycerides were obtained, "A," the greater part of the whole, consisting of almost completely neutral fat, and "B," the product recovered from the ether extraction of the alkaline and aqueous washings, smaller in quantity than "A" and still somewhat contaminated with acidic material. The ether-extracted washings were treated with mineral acid and the acidic oxidation products were recovered: the acid value of the latter was observed and employed to estimate acidic products still present in fraction "B," on the assumption that the acid value of acidic

products in "B" was approximately the same as that of those removed during the purification.

These data suffice to determine the fully-saturated glyceride content of the original fat; from the saponification equivalents of the purified fully-saturated glycerides "A" and of the original butter-fat the molar percentages of fully-saturated glycerides follow, whilst, knowing the proportion of unsaturated acids present in the original fat from the earlier detailed analyses [1930, p. 1005], it is also possible to estimate the molar proportions of saturated acid present in the mixed saturated-unsaturated (and completely unsaturated) glycerides—the "association-ratio."

The results thus obtained for butter-fats I–V are collected in Table I.

Table I. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) *Analytical data.*

			Purification of crude fully-saturated glycerides							
				"A"		"B"		Acidic products		
No.	Weight taken g.	Crude F.S.G. g.	Weight taken g.	g.	Acid No.	g.	Acid No.	g.	Acid No.	F.S.G. % (weight)
I	601·9	182·4	61·8	42·2	0·5	9·5	8·8	9·9	153·9	25·4
II	606·5	248·0	244·2	204·6	0·3	26·7	6·5	12·9	170·0	38·5
III	501·2	183·3	181·3	150·8	0·4	24·6	9·9	5·9	149·5	35·05
IV	202·1	57·8	57·2	32·6	0·6	17·5	14·3	7·2	190·6	24·35
V	303·5	120·9	120·9	102·3	1·2	11·8	14·4	6·8	258·3	37·4

(ii) *Summary of observed values.*

		Acids in whole fat		Fully-saturated glycerides		"Association ratio" in non-fully-saturated part Mols. saturated acid per mol. unsaturated acid
No.	Iodine value	Saturated % (mol.)	Unsaturated % (mol.)	Weight %	Mols. %	
I	41·3	63·0	37·0	25·4	29·1	0·92
II	31·6	72·4	27·6	38·5	41·3	1·11
III	34·8	70·0	30·0	35·05	38·2	1·07
IV	41·6	61·9	38·1	24·35	27·2	0·94
V	34·5	70·2	29·8	37·4	39·6	1·07

Fatty acid compositions of fully-saturated glycerides of butter-fats I–V.

The purified fully-saturated glycerides "A" were converted into their mixed fatty acids and analysed as described in our former communication [1930], the operations being somewhat simplified by the absence of unsaturated acids and consequent omission of the lead salt separation of the non-volatile acids. An illustration of the results of this method of analysis in detail as applied to the whole acids of butter-fat IV was given in the paper referred to, and in the present cases only the final amounts of each acid as determined in the analyses are recorded.

Direct analysis of the saturated fatty acids combined in the acidic products of oxidation of mixed saturated-unsaturated glycerides cannot be effected owing to the presence of nonoic and azelaic acids as products of oxidation,

Table II. *Summarised analytical data for fatty acids from fully-saturated glycerides of butter-fats I–V.*

Acid	Volatile acids g.	Acids non-volatile in steam g.	Total g.	% (weight) (excluding unsaponifiable matter)	% (mol.) (excluding unsaponifiable matter)
		Fully-saturated glycerides of butter-fat I.			
	8·39	92·7	101·09		
Butyric	4·70	—	4·70	4·7	11·7
Caproic	2·80	—	2·80	2·8	5·3
Caprylic	0·67	0·76	1·43	1·4	2·2
Capric	—	3·28	3·28	3·3	4·2
Lauric	—	4·79	4·79	4·7	5·2
Myristic	—	13·69	13·69	13·6	13·2
Palmitic	—	50·40	50·40	50·0	43·1
Stearic	—	19·60	19·60	19·5	15·1
Unsap.	0·22	0·18	0·40	—	—
		Fully-saturated glycerides of butter-fat II.			
	15·45	175·2	190·65		
Butyric	7·21	—	7·21	3·8	9·2
Caproic	5·94	—	5·94	3·1	5·8
Caprylic	1·75	1·82	3·57	1·9	2·8
Capric	0·55	10·08	10·63	5·6	6·9
Lauric	—	19·73	19·73	10·4	11·1
Myristic	—	40·72	40·72	21·4	20·1
Palmitic	—	80·54	80·54	42·3	35·4
Stearic	—	21·87	21·87	11·5	8·7
Unsap.	—	0·44	0·44	—	—
		Fully-saturated glycerides of butter-fat III.			
	10·72	114·6	125·32		
Butyric	4·67	—	4·67	3·7	9·2
Caproic	4·29	—	4·29	3·4	6·4
Caprylic	1·04	1·53	2·57	2·1	3·1
Capric	0·72	5·54	6·26	5·0	6·3
Lauric	—	7·40	7·40	5·9	6·4
Myristic	—	25·67	25·67	20·5	19·6
Palmitic	—	53·20	53·20	42·5	36·1
Stearic	—	20·63	20·63	16·5	12·6
Arachidic	—	0·50	0·50	0·4	0·3
Unsap.	—	0·13	0·13	—	—
		Fully-saturated glycerides of butter-fat IV.			
	9·12	95·45	104·57		
Butyric	4·795	—	4·795	4·6	11·4
Caproic	2·815	—	2·815	2·7	5·1
Caprylic	0·88	0·94	1·82	1·7	2·7
Capric	0·63	3·71	4·34	4·2	5·3
Lauric	—	5·75	5·75	5·5	6·0
Myristic	—	16·41	16·41	15·7	15·1
Palmitic	—	48·17	48·17	46·2	39·5
Stearic	—	20·20	20·20	19·4	14·9
Unsap.	—	0·27	0·27	—	—
		Fully-saturated glycerides of butter-fat V.			
	13·51	157·3	170·81		
Butyric	7·63	—	7·63	4·5	11·2
Caproic	4·09	—	4·09	2·4	4·6
Caprylic	1·51	2·22	3·73	2·2	3·4
Capric	—	6·84	6·84	4·0	5·1
Lauric	—	8·21	8·21	4·8	5·3
Myristic	—	26·27	26·27	15·4	14·9
Palmitic	—	79·11	79·11	46·5	39·9
Stearic	—	34·34	34·34	20·2	15·6
Unsap.	0·28	0·31	0·59	—	—

but the approximate composition of the unsaturated glycerides can be estimated by the difference between the respective acids present in the fully-saturated portion and in the whole fat as in Table III.

Table III. *General composition (weight) of butter-fats I–V.*

	Whole fat	Fully-saturated	Non-fully-saturated
	Butter-fat I.		
	100 g.	25·4 g.	74·6 g.
Glyceryl residue $C_3H_5\equiv$	5·05	1·4	3·65
Butyric acid	2·95	1·1	1·85
Caproic ,,	1·65	0·65	1·0
Caprylic ,,	1·5	0·35	1·15
Capric ,,	2·0	0·8	1·2
Lauric ,,	3·25	1·15	2·1
Myristic ,,	6·55	3·25	3·3
Palmitic ,,	27·5	12·0	15·5
Stearic ,,	7·2	4·7	2·5
Arachidic ,,	0·85	—	0·85
Oleic ,,	38·0	—	38·0
Linoleic ,,	3·5	—	3·5
	Butter-fat II.		
	100 g.	38·5 g.	61·5 g.
Glyceryl residue $C_3H_5\equiv$	5·2	2·15	3·05
Butyric acid	3·2	1·4	1·8
Caproic ,,	1·9	1·15	0·75
Caprylic ,,	1·05	0·7	0·35
Capric ,,	3·05	2·0	1·05
Lauric ,,	6·9	3·75	3·15
Myristic ,,	16·2	7·75	8·45
Palmitic ,,	25·6	15·4	10·2
Stearic ,,	4·55	4·2	0·35
Oleic ,,	30·05	—	30·05
Linoleic ,,	2·3	—	2·3
	Butter-fat III.		
	100 g.	35·05 g.	64·95 g.
Glyceryl residue $C_3H_5\equiv$	5·2	1·95	3·25
Butyric acid	3·4	1·2	2·2
Caproic ,,	1·9	1·15	0·75
Caprylic ,,	1·6	0·7	0·9
Capric ,,	3·5	1·65	1·85
Lauric ,,	6·2	1·95	4·25
Myristic ,,	10·0	6·8	3·2
Palmitic ,,	24·8	14·1	10·7
Stearic ,,	7·9	5·45	2·45
Arachidic ,,	1·1	0·1	1·0
Oleic ,,	31·0	—	31·0
Linoleic ,,	3·4	—	3·4
	Butter-fat IV.		
	100 g.	24·35 g.	75·65 g.
Glyceryl residue $C_3H_5\equiv$	5·05	1·35	3·7
Butyric acid	3·1	1·1	2·0
Caproic ,,	1·2	0·6	0·6
Caprylic ,,	1·15	0·4	0·75
Capric ,,	2·1	1·0	1·1
Lauric ,,	3·8	1·3	2·5
Myristic ,,	9·9	3·6	6·3
Palmitic ,,	24·8	10·6	14·2
Stearic ,,	6·2	4·4	1·8
Oleic ,,	38·8	—	38·8
Linoleic ,,	3·9	—	3·9

Table III (*contd.*).

	Whole fat	Fully-saturated	Non-fully-saturated
	Butter-fat V.		
	100 g.	37·4 g.	62·6 g.
Glyceryl residue $C_3H_5\equiv$	5·15	2·05	3·1
Butyric acid	3·3	1·6	1·7
Caproic ,,	1·6	0·85	0·75
Caprylic ,,	1·25	0·8	0·45
Capric ,,	2·9	1·4	1·5
Lauric ,,	3·85	1·7	2·15
Myristic ,,	10·55	5·45	5·1
Palmitic ,,	25·9	16·45	9·45
Stearic ,,	10·9	7·1	3·8
Arachidic ,,	0·6	—	0·6
Oleic ,,	29·75	—	29·75
Linoleic ,,	4·25	—	4·25

Discussion.

As already mentioned, we desire to review here the data for all the cow butter-fats which have now been submitted to examination by these methods, *i.e.* the present series of five specimens together with two former (market) samples of New Zealand butter and the recently-investigated specimen of Indian cow ghee. We thus have at disposal two Berkshire pasture-fed (late autumn and early summer), one Indian pasture-fed and three New Zealand (probably all pasture-fed) butters, with two other Berkshire butters from cows on winter diet supplemented by specific oil-cakes.

We find that our results lead to the consideration of two separate aspects of the glyceride structure of the butter-fats, namely, (i) the general distribution of unsaturated acids throughout the glycerides, which appears to be independent of the particular saturated acid components of the fat; and (ii) certain regularities in the occurrence of the saturated acid components in all the fats except those two in which added oil-cake formed a prominent part of the diet.

General distribution of unsaturated acids in the butter glycerides.

The data for all eight fats show consistently that the content of fully-saturated glycerides is, within close limits, simply a function of the relative proportions of saturated and unsaturated acids in the whole fat, irrespective of the nature of the saturated acids (the unsaturated acids are throughout almost constant in composition, *viz.* about 90 % oleic and about 10 % linoleic). In this respect the butter-fats resemble such other animal fats (beef and mutton tallows, lards, rabbit-fat) as have been examined, and also vegetable "pericarp" fats such as palm oil or stillingia tallow; all these are also not far removed in fully-saturated glyceride content (i) from glycerides of the same degree of unsaturation prepared synthetically by heating glycerol with a slight excess of the mixed fatty acids [*cf.* Bhattacharya and Hilditch, 1930], or

(ii) from that calculated on the assumption that the content of fully-saturated glycerides in mixed glycerides of saturated and unsaturated acids is proportional to the cube of the concentration of the total saturated acids. It will be remembered that vegetable seed-fats appear to be elaborated on entirely different and more "evenly distributed" lines [Collin and Hilditch, 1929].

The present series covers a relatively wide range of variation in the milk-fats from a single animal species and affords a more stringent comparison than has hitherto been possible of the relation of fully-saturated glyceride content to total saturated acid content in different specimens of a single type of fat. In Table IV will be found the iodine values and molar percentages[1] of saturated acids in the original butter-fats, together with the observed molar percentages of fully-saturated glycerides and (i) the latter calculated on the basis of proportionality to the cube of the total saturated acid present and (ii) the molar percentages of fully-saturated glycerides in synthetic mixed glycerides taken from the curve given by Bhattacharya and Hilditch [1930]; the "association ratio," or mols. of saturated acid present per mol. of unsaturated acid, in the non-fully-saturated glycerides is added in the final column.

Table IV. *Relationship between fully-saturated glyceride content and total saturated acid content of butter-fats.*

			Fully-saturated glyceride content			
Butter-fat (*cf.* p. 507)	Iodine value	Total saturated acids % (mols.)	Observed % (mols.)	Calculated ($y \propto x^3$) % (mols.)	From synthetic glyceride curve (B and H) % (mols.)	"Association ratio" (observed)
II	31·6	72·4	41·3	38·0	34·0	1·11
V	34·5	70·2	39·6	34·5	30·5	1·07
III	34·8	70·0	38·2	34·2	30·2	1·07
CG	36·0	67·9	33·7	31·5	27·5	1·07
A	38·0	67·3	33·8	30·5	26·5	1·04
B	39·4	66·1	31·5	28·7	25·0	1·03
I	41·3	63·0	29·1	24·5	21·5	0·92
IV	41·6	61·9	27·2	23·3	20·5	0·94

These relations are shown, probably more clearly, in Fig. 1, in which the appropriate portions of Bhattacharya and Hilditch's "synthetic" glyceride curve (unbroken line) and of the "calculated" curve (dotted) are plotted on an enlarged scale together with the observed values for the eight butter-fats. All the butter-fats contain rather more fully-saturated glycerides than would be the case if the fully-saturated glyceride content varied as the cube of the total saturated acids present, whilst, at this range of total unsaturation, the

[1] Throughout this discussion we are concerned with the relative number of molecules (or, perhaps, equivalents) of the various glycerides and fatty acids—the weight percentages usually employed are of little use in interpreting the structure of a complex mixture of glycerides of acids which differ widely (88 to 284) in molecular weight.

synthetic glycerides contain somewhat less fully-saturated components than the amount corresponding with this relationship. The relative regularity in the alteration of fully-saturated glyceride content with total unsaturation in the case of the butter-fats is perhaps more striking than the parallelism with either of the curves in question. The corresponding alteration in the proportions of saturated and unsaturated acids in the non-fully-saturated glycerides (Table IV, final column) should also be noted[1].

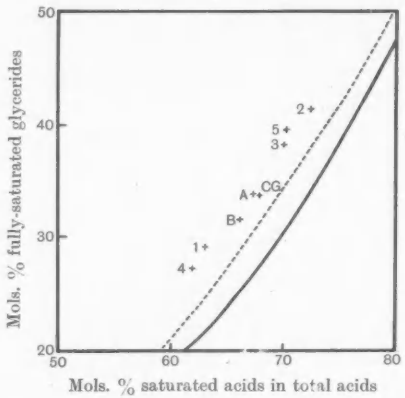

Fig. 1.

These data are considered to show that, as between saturated and unsaturated acids, the constitution of the mixed glycerides in butter-fat is determined by the relative concentration of these classes of acid; this proportion being given in any particular case, the mode of assemblage into triglyceride molecules appears to follow according to some fixed rule. Although the experimental methods only permit of differentiation between saturated and unsaturated acids, there is no reason to suppose that, normally, the case is different as between one saturated acid and another.

As regards the general composition of the non-fully-saturated glycerides of the eight fats, the molar "association ratios" in the final column of

[1] By way of contrast, the corresponding available data for the two seed-fats so far examined [Collin and Hilditch, 1929] in this region of relative unsaturation may be recalled:

Fat	Iodine value	Total saturated acids % (mols.)	F.S.G. content % (mols.)	"Association ratio"
Cacao butter	37·1	59·8	2·5	1·4
Illipé tallow	32·3	62·8	4·5	1·55

In seed-fats, so long as there is sufficient saturated acid present, there is a tendency towards a constant association of about 1·4 mols. saturated with 1 mol. of unsaturated acid in the "mixed" glycerides and, at this particular state of relative unsaturation, the content of fully-saturated glycerides is exceedingly small.

Table IV permit limiting values to be alculated for the possible proportions of either mono-unsaturated di-sat rated and di-unsaturated monosaturated glycerides, or mono-unsaturated di-saturated and tri-unsaturated glycerides which may be present; in the absence of any analytical method which will allow the amount of any one of these classes (*e.g.* triolein) to be determined, this is as far as we can go at present. On the other hand, there is little or no evidence that triolein occurs in any great amount in butter-fat, and considerable indirect evidence against this possibility [*cf.* Amberger, 1918]; and very probably the minimum limits for mono-unsaturated derivatives and the maxima for the di-unsaturated class given in Table V are not far removed from the actual proportions present.

Table V. *Limiting values for the molar percentages of the four types of mixed glycerides in butter-fats.*

			Glycerides (mols. %)			
Butter	Iodine value	"Association ratio"	Fully - saturated	Mono-unsaturated-di-saturated	Di-unsaturated-mono-saturated	Tri-unsaturated
II	31·6	1·11	41·3	33·9–46·3	24·8–0	0–12·4
V	34·5	1·07	39·6	33·3–46·8	27·1–0	0–13·6
III	34·8	1·07	38·2	34·0–47·9	27·8–0	0–13·9
CG	36·0	1·07	33·7	36·5–51·4	29·8–0	0–14·9
A	38·0	1·04	33·8	35·0–50·6	31·2–0	0–15·6
B	39·4	1·03	31·5	35·8–52·1	32·7–0	0–16·4
I	41·3	0·92	29·1	31·0–51·0	39·9–0	0–19·9
IV	41·6	0·94	27·2	33·0–52·9	39·8–0	0–19·9

Whilst these general characteristics of butter-fat glyceride structure may prove to be of some importance with regard to the biochemical elaboration of milk-fats, they also have a practical bearing on the variation in the "melting point" or softness of butters. It is evident that increase in oleic acid content of butter, according to the above data, will be reflected in a disproportionate increase in softness, since not only is the ratio of unsaturated to saturated acids increased in the saturated-unsaturated mixed glycerides, but the quantity of the latter increases considerably with augmentation of the oleic acid. For example, in 100 mols. of fat, butter-fat IV contains about 73 mols. of mixed glycerides in which the unsaturated acids are in excess of the saturated, whereas butter-fat II contains only about 59 mols. of mixed glycerides in which there are 11 mols. of saturated acid for every 10 of unsaturated. Whilst, therefore, the fully-saturated glycerides (which are of course the highest-melting components of butter) have been reduced by 14 mols. in every 100 of total fat, the mixed glycerides contain relatively more combined oleic acid, again leading to the production of components of lower melting point. These data also illustrate the obvious reasons for the varying differences which obtain between the melting or softening point of most natural fats and the corresponding (and usually much higher and sharper) melting points of their respective mixed fatty acids; the melting points of individual simple triglycerides being, on the other hand, of the same order

and usually somewhat higher than those of the fatty acid from which they are derived [Joglekar and Watson, 1930].

Distribution of the individual fatty acids in the butter-glycerides.

In order to discuss several features of this aspect of the glyceride structure it will be necessary to refer to the molar percentages of each acid combined in the whole fat, the fully-saturated glycerides and the remaining glycerides; these are collected in Tables VI, VII and VIII. The butter-fats are arranged in increasing order of total unsaturation except that, since the fats II and III (from cows whose food contained large amounts of specific oil-cakes) show certain differences from the remaining (pasture-fed) fats, the data for samples II and III are placed at the end of each table.

Table VI. *Molar distribution of fatty acids in the whole fat.*

Fat ...	V	CG	A	B	I	IV	II	III
Butyric acid	9·2	6·9	9·2	8·4	8·4	8·9	9·0	9·6
Caproic ,,	3·4	4·0	3·7	3·9	3·5	2·7	3·9	3·0
Caprylic ,,	2·2	2·2	1·4	1·3	2·7	2·0	1·7	2·8
Capric ,,	4·2	4·9	2·7	2·8	2·9	3·0	4·3	5·1
Lauric ,,	4·7	6·7	3·7	4·6	4·1	4·7	8·3	7·5
Myristic ,,	11·5	10·9	10·2	11·0	7·2	10·9	17·2	10·7
Palmitic ,,	25·0	26·8	25·7	26·2	27·1	24·3	24·1	23·7
Stearic ,,	9·5	5·5	10·2	7·1	6·4	5·4	3·9	6·7
Arachidic ,,	0·5	—	0·5	0·8	0·7	—	—	0·9
Oleic ,,	26·1	28·4	28·9	30·8	33·9	34·6	25·7	27·0
Linoleic ,,	3·7	3·7	3·8	3·1	3·1	3·5	1·9	3·0

Table VII. *Molar distribution of fatty acids in the fully-saturated glycerides.*

Fat	V	CG	A	B	I	IV	II	III
Molar % F.S.G. ...	39·6	33·7	33·8	31·5	29·1	27·2	41·3	38·2
Butyric acid	11·2	11·2	11·0	10·5	11·7	11·4	9·2	9·2
Caproic ,,	4·6	5·1	6·5	4·9	5·3	5·1	5·8	6·4
Caprylic ,,	3·4	0·5	1·8	5·0	2·2	2·7	2·8	3·1
Capric ,,	5·1	4·4	3·3	3·1	4·2	5·3	6·9	6·3
Lauric ,,	5·3	6·1	4·1	4·7	5·2	6·0	11·1	6·4
Myristic ,,	14·9	15·5	17·9	17·0	13·2	15·1	20·1	19·6
Palmitic ,,	39·9	43·0	39·6	39·3	43·1	39·5	35·4	36·1
Stearic ,,	15·6	14·2	15·8	15·2	15·1	14·9	8·7	12·6
Arachidic ,,	—	—	—	0·3	—	—	—	0·3

Table VIII. *Molar distribution of fatty acids in the mixed saturated-unsaturated glycerides.*

Fat	V	CG	A	B	I	IV	II	III
Molar % M.S-U ...	60·4	66·3	66·2	68·5	70·9	72·8	58·7	61·8
Butyric acid	7·9	4·8	8·3	7·3	7·2	7·8	8·6	9·6
Caproic ,,	2·6	3·4	2·3	3·3	2·9	2·2	2·7	2·5
Caprylic ,,	1·3	3·1	1·1	—	2·8	1·8	1·0	2·4
Capric ,,	3·6	5·1	2·3	2·6	2·4	2·2	2·4	4·1
Lauric ,,	4·4	7·0	3·4	4·7	3·7	4·3	6·4	8·2
Myristic ,,	9·2	8·6	6·4	8·4	5·0	9·4	15·1	5·4
Palmitic ,,	15·0	18·6	18·9	20·1	20·9	18·7	16·3	16·1
Stearic ,,	5·6	1·1	7·5	3·3	3·1	2·2	0·5	3·3
Arachidic ,,	1·0	—	0·7	1·1	1·0	—	—	1·2
Oleic ,,	43·2	42·7	43·4	44·8	46·7	46·7	43·7	42·5
Linoleic ,,	6·2	5·6	5·7	4·4	4·3	4·7	3·3	4·7

Further, we have found it convenient to compare the component acids in the following groups: (i) the lower acids from butyric to lauric inclusive, (ii) myristic and palmitic; and (iii) the total amount of acids containing 18 carbon atoms in the molecule (the very small quantities of C_{20} acids recorded in some of the analyses are included with this group). The molar percentages of these groups present in each fat and in its two subdivisions are shown in Table IX.

Table IX. *Molar distribution of butyric-lauric, myristic-palmitic, and C_{18} acids in the butter-fats.*

	Fat	V	CG	A	B	I	IV	II	III
Section	Acids								
Whole fat	Butyric-lauric	23·7	24·7	20·7	21·0	21·6	21·3	27·2	28·0
	Myristic-palmitic	36·5	37·7	35·9	37·2	34·3	35·2	41·3	34·4
	C_{18} acids	39·8	37·6	43·4	41·8	44·1	43·5	31·5	37·6
Fully saturated	Butyric-lauric	29·6	27·3	26·7	28·2	28·6	30·5	35·8	31·4
	Myristic-palmitic	54·8	58·5	57·5	56·3	56·3	54·6	55·5	55·7
	Stearic	15·6	14·2	15·8	15·5	15·1	14·9	8·7	12·9
Saturated-unsaturated	Butyric-lauric	19·8	23·4	17·4	17·9	19·0	18·3	21·1	26·8
	Myristic-palmitic	24·2	27·2	25·3	28·5	25·9	28·1	31·4	21·5
	C_{18} acids	56·0	49·4	57·3	53·6	55·1	53·6	47·5	51·7
	(Oleic-linoleic	49·4	48·3	49·1	49·2	51·0	51·4	47·0	47·2)

The six butter-fats from cattle on normal diets. The cows from various districts which were fed on pasture (or on pasture with roots, *etc.*, but no added oil-cake) yielded butters which show much similarity in many features of their glyceride structure.

(i) In the total fatty acids of each whole butter-fat (Table VI), the amounts of (*a*) butyric-lauric, (*b*) myristic-palmitic and (*c*) stearic, oleic and linoleic acids tend severally towards more or less constant values. This approximate constancy extends to some of the individual acids, especially palmitic, myristic and perhaps butyric; but the amount of stearic acid is more variable. Yet the total amount of C_{18} acids is of much the same order throughout, variations due to the substantially different amounts of unsaturated acids in the butters being counterbalanced partly by variation in the amount of stearic acid, partly by the gross effect of minor variations in all the other saturated acids.

(ii) Although, as the total unsaturation in the fats varies, the amount of fully-saturated glycerides differs widely throughout the series, their composition (Table VII) is remarkably constant; especially noteworthy is the regular proportion of stearic acid ($15 \pm 0{\cdot}8$ mols. %) in contrast to its variability (5–10 mols. %) in the whole fats. All the saturated acids are present in both fully- and non-fully-saturated parts of the fats, but the tendency noticed by Hilditch and Jones [1929] for the lower acids to associate slightly more than the higher saturated acids with the unsaturated acids is confirmed by the present more extended results.

The inference can fairly be drawn, for these "normal diet" butters, that,

whilst the whole fats vary in unsaturation over a range of 34·5–41·3 in iodine value (61·9–70·2 mols. % total saturated acids), the composition of the fully-saturated glycerides (which comprise from 27 to 40 mols. % of the whole fat) is in every case much the same.

(iii) In the non-fully-saturated glycerides (Table VIII), some approximation to constancy in the molar content of the three groups of acids mentioned follows automatically from the general tendencies noted in (i) and (ii) above. The proportion of unsaturated acids (Table IX, last line) increases slowly, but on the whole steadily, with the total unsaturation of the butter-fats as a whole, but in less than direct proportion to the molar percentage of unsaturated acids present in the whole fat.

(iv) The degree of concordance shown in the totals of the molar contents of butyric-lauric acids is of special interest in view of the importance of these acids as the characteristic acids of milk-fats. In the whole fats this figure appears to tend to a constant value of about 21–22, four of the six fats giving values between 20·7 and 21·6 (Table VI). The maximum variation in the combined butyric-lauric acid molar content of the six butter-fats is, indeed, much less than that in the corresponding Reichert-Meissl values; it is not unlikely that, with a reasonably rapid analytical method for the determination of combined butyric-lauric acid content, this figure would be a more certain index for milk-fats than the Reichert-Meissl value. We are endeavouring to ascertain whether a simplified procedure can be devised by means of which a measure of the total butyric-lauric acid content may be more readily obtained than by employment of the full "fractionation" analysis.

We might not have felt justified, at this stage, in stressing some of the foregoing likenesses in glyceride structure of these butters except for the fact that an examination of a number of beef tallows from different sources, now being made by Mr A. Banks in this laboratory, is yielding results of a similar and equally concordant nature. In the tallow investigation, an account of which it is hoped to publish at an early date, the number of individual component acids to be considered is much less than in butters and the regular features which we have been discussing stand out more sharply in consequence. At the same time, notwithstanding the complexity of the fatty acid mixture in butters, inspection of the foregoing tables on the lines suggested by this more recent work on the body-fats has shown that similar influences operate in the case of the milk-fats from pasture-fed sources.

It is interesting to find, further, that the combined molar contents of myristic and palmitic acids in the total butter-fats, and in their fully-saturated portions, are only a few mols. % lower than the corresponding average values for beef tallows. In other words, the molar contents of C_{18} acids in the tallows (*a*) as a whole or (*b*) in their fully-saturated components are respectively within a few units of the molar contents of all the acids, other than myristic and palmitic, (*a*) in butter-fat or (*b*) in its fully-saturated components. This naturally suggests that the lower fatty acids characteristic of butter are

elaborated at the expense of acids of the stearic series. We are not disposed to assert that the lower fatty acids present in butter necessarily result directly from stearic or oleic acid, but the data which we have put forward are definitely consistent with the view that a precursor of stearic or oleic acid in a tallow may, in the course of milk-fat metabolism, appear instead as a lower fatty acid, with the proviso that only one molecule of any of the lower acids is produced in milk-fat for each molecule of stearic (oleic) acid which otherwise would have resulted in a body-fat. We also consider that the present data confirm our former view that palmitic and (possibly) myristic acids stand apart from the remaining butter acids in many respects, and in particular we find it difficult at present to suppose that the sources of palmitic acid and of oleic, stearic, *etc.*, acids in the natural fats can be identical.

At first glance it may seem difficult to reconcile these regularities in glyceride structure with the variations in the component fatty acids of the same series of butter-fats which we discussed in our previous paper and which we showed to be somewhat large, and due more to conditions such as the change from outdoor to indoor life, general type of diet, or seasonal changes of temperature than to added fats given in the diet. It was pointed out, however [1930, p. 1110], that the chief variations due to such causes were in the content of unsaturated acids and of stearic acid, *i.e.* on the whole, in the balance between stearic, oleic and linoleic acids. Since the amount of fully-saturated glycerides depends on the relative amount of unsaturated acids present in the whole fat, this is a variable, indeed almost the only markedly variable, feature in the glyceride structure of the butters; for the rest, the assemblage of fatty acids into glycerides evidently operates in the same way whatever may be the individual acids concerned (the manner being very similar for such animal fats and vegetable "pericarp" fats as have been studied, but different in the case of vegetable seed-fats). Thus, when the factor due to variable unsaturation (the varying fully-saturated glyceride content) has been taken into account, close similarities are revealed in the respective fully-saturated and non-fully-saturated components of the butters—the constancy of the stearic acid content of the fully-saturated portions being, perhaps, the most illuminating feature.

The two butter-fats from cows whose diet included specific oil-cakes.

By way of contrast, the differences due to inclusion in the diet of coconut or soya bean cake appear more clearly from the component glyceride structures than from simple consideration of the total fatty acids of butters II and III [1930, p. 1111]; this will be seen by comparing the data in Tables VI–IX for Nos. II and III with those for the other six butters.

(i) *Coconut cake in diet (butter II).* It was pointed out previously [1930] that the only distinctive features in the total acids of butter-fat II were increased amounts of myristic and lauric acids, with, however, less augmentation in the lauric, capric and caprylic acids than might have been anticipated

in view of the high lauric acid and appreciable capric and caprylic acid contents of coconut oil.

In the fully-saturated components of butter-fat II, however, the relative differences are more sharply defined: the lauric acid molar content (Table VII) is 11 (instead of about 5–6) and the myristic acid molar content is 20 (against 15–17). Since dilauromyristin is probably the major component of coconut oil [Collin and Hilditch, 1929], the figures suggest that to some extent this glyceride has passed through directly into the milk-fat, an inference which is supported by consideration of the butyric-lauric acid contents of the fully-saturated and non-fully-saturated parts of the fat.

The butyric-lauric acid content (Table IX) as a whole is much higher (35·8 mols. %) than in the fully-saturated components of the six "normal" butters (27–30 mols. %), whilst the palmitic and stearic acid contents are correspondingly low; the latter (9 mols. %) is in sharp contrast to the steady value of about 15 mols. % observed in the pasture-fed butters (fully-saturated glycerides). Thus the amounts of the individual components of the fully-saturated part of butter-fat II (except capric and caprylic acids) differ distinctly from those in the six butter-fats discussed above. On the other hand, the combined butyric-lauric acid content (21·1 mols. %) of the non-fully-saturated glycerides of butter-fat II is only slightly higher than the corresponding values for the normal fats—indeed the only notable divergence from the normal is an increase in the myristic acid and a minor increase in lauric acid. Consequently, practically all the differences in the butter-fat, caused by feeding coconut-fat (which contains about 85 % of fully-saturated components) to the cows, have been traced to variations from the normal composition in the fully-saturated part of the butter. Whilst, therefore, these more intimate analyses support our earlier conclusion that the greater part of the characteristic components of the coconut-fat have not appeared in the milk-fat, they clearly indicate the location of certain portions of the typical coconut fully-saturated glycerides which have, apparently, passed unchanged into the milk.

(ii) *Soya bean cake in diet* (*butter III*). It has already been emphasised [1930] that the relatively large amount of linoleic acid in soya bean oil has little appreciable effect on the content of linoleic and oleic acids in the butter-fat, which is of the normal order. Examination of the component glycerides reveals (Table IX) that in butter III the butyric-lauric acid content of the non-fully-saturated components (26·8 mols. %) is much higher than the average value for the six "normal" butters (18–20 mols. %); myristic and stearic acids (Table VIII) are about normal and palmitic acid (16·1 mols. %) is definitely below the normal (18–20 mols. %). On the other hand, the glyceride structure of the fully-saturated part (except for a somewhat large content of myristic acid) is not far from the normal range of the six pasture-fed butters.

Thus whilst ingestion of the relatively highly saturated coconut-fat led

to variation from the normal in the character of the fully-saturated components of butter-fat II, the diet including soya bean oil (nearly all of which consists of unsaturated glycerides) has caused differences from the normal which are for the most part confined to the non-fully-saturated glycerides of butter-fat III. In the latter case, however, the observed differences do not involve any increase in the proportion of linoleic acid (the major component of soya bean oil) but are confined for the most part to augmentation of the butyric-lauric acid group; thus suggesting that the latter may, in part, be metabolic products from the soya bean oil acids which remain combined in the mixed saturated-unsaturated glycerides.

It will be recalled that the New Zealand December pasture-fed butter-fat V, which has almost the same iodine value as butter-fat III from the soya bean cake diet, resembles the latter in much of its total fatty acid composition [Hilditch and Sleightholme, 1930, p. 1110]; but the above study of the glyceride structure reveals the differences which in reality exist between the two fats. Indeed, unless investigation of glyceride structure be undertaken it appears that sufficient data cannot be provided for the adequate consideration of milk-fat metabolism.

Summary.

Four English butter-fats from members of the same herd of cows fed on various diets and a New Zealand pasture-fed (December) butter-fat (the total fatty acid compositions of which have been previously discussed) have been oxidised in order quantitatively to determine their content of fully-saturated glycerides, and the fatty acids of the latter have been analysed in detail. The results obtained, with similar data for two other New Zealand butter-fats and an Indian cow ghee, are discussed with reference to the general glyceride structure of butter-fats.

The six butters from cows on "normal" (usually pasture-fed) diets show many features in common, whilst in two cases in which specific fatty oils had been fed to the cows the results stand apart in certain respects; but all eight fats conform to the usual rule (in animal fats) that the content of fully-saturated glycerides is a function of the proportion of the total saturated acids present in the mixed acids of the whole fat.

In the "normal" butters, moreover, the component acids of the fully-saturated parts are present in approximately the same quantitative amounts, the composition of the fully-saturated glycerides tending to be the same whatever the original unsaturation of the butter as a whole. Similar concordances are revealed in the combined butyric-lauric acid contents, and in the combined myristic-palmitic acid contents, of the whole fats and of their two divisions, fully- and non-fully-saturated glycerides, whilst the content of unsaturated acids in the non-fully-saturated glycerides increases steadily and slowly with increase of unsaturation in the fats as a whole.

When coconut-fat (a relatively saturated fat) formed part of the diet, the

fully-saturated part of the butter showed a marked increase in butyric-lauric acid content, but for the most part the composition of the non-fully-saturated glycerides was normal. With a soya bean (predominantly unsaturated) oil diet the structure of the butter glycerides was different: the fully-saturated components were not far removed from the normal in composition, but the lower acids of the non-fully-saturated portion were present in excess of the usual proportion.

Study of the glyceride structure of these eight butters, whilst confirming the general rules which have been observed to connect the amount of fully-saturated glycerides with the degree of unsaturation of animal fats, thus reveals influences due to diet more clearly than the consideration of the component fatty acids of the whole fats, and affords some additional evidence as to the mode of utilisation of ingested fat in milk-fat metabolism.

We have again to express our grateful appreciation for the assistance given to us by Captain Golding and the National Institute for Research in Dairying and to thank Messrs Lever Brothers, Ltd., for a Research Studentship held by one of us (J.J.S.) during the course of the work.

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LXII. THE EFFECT OF HYDROGEN ION CONCENTRATION ON SOME OXIDATIONS BY *B. COLI COMMUNIS*.

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The effect of treatment with toluene on the aerobic and anaerobic oxidations of various substrates has been noted in a previous communication [Cook, 1930]. The present paper is the outcome of experiments which were undertaken to investigate the most favourable conditions of hydrogen ion concentration for the aerobic and anaerobic oxidations of formate, succinate and lactate. Apart from the immediate practical value of these experiments, the results are of interest in relation to the kinetics of the action of oxidative enzymes and the interaction of the dehydrogenases and oxygenases in the bacterial cell.

The p_H-activity curves of the oxidases have not been studied in such great detail as those of the hydrolytic enzymes. For a summary of work on the latter Haldane's monograph [1930] should be consulted. Ohlsson [1921] made a very careful study of extracted muscle succinoxidase, the results of which in relation to the present work will be discussed later. Quastel and Whetham [1924] investigated the effect of p_H on the dehydrogenation of succinate by normal *B. coli*. Dixon and Thurlow [1924] and Bernheim [1928] studied the p_H-activities of xanthine oxidase and the potato aldehyde oxidase respectively. In all the above instances, with the exception of one experiment by Bernheim who used nitrate, methylene blue was used as the hydrogen acceptor. There appears to be little work on the oxidases in which oxygen either activated or not is involved. Hare [1928] made an interesting study of the p_H-activity curve of tyramine oxidase in which unactivated oxygen is involved. Cook and Stephenson [1928] studied the effect of p_H on the oxidation of lactate by activated oxygen by normal *B. coli*. References to peroxidase, catalase and some other oxidases are given in Haldane's monograph.

It had been noted earlier [Cook, 1930] that above a temperature of about 30° the rate of oxygen reduction appeared to be the limiting factor in the aerobic oxidation of formate and lactate. The aerobic oxidation of these substrates was therefore studied at 16° and 40°. The anaerobic experiments in which the reduction of methylene blue in the Thunberg tube was observed and which are a measure solely of the dehydrogenases were carried out at 40°.

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From the earlier results it was shown that the reaction velocity increased in a linear manner with increase of temperature until destruction of the enzyme occurred at a temperature of about 50°. Some experiments carried out at a lower temperature showed that the shape of the p_H-activity curves remained substantially the same as that of those at 40°.

Experimental.

The bacterial suspension used for these experiments was derived from a strain of *B. coli communis* Escherich obtained from the National Collection of Type Cultures. Some experiments were carried out on a strain obtained from the Pathology Department, Cambridge, but apart from slight differences in the velocities of respiration no significant differences were noted in the shapes of the curves. The method by which the stock preparation is prepared has been described previously [Cook, 1930]. This preparation retains its activity for several weeks if kept in the refrigerator, but if left in the open autolysis sets in and it rapidly deteriorates. The effect is most marked on the reduction of oxygen. The reduction of methylene blue especially by formate and lactate shows only a slight falling off in activity. From the stock preparation of bacteria the toluene-treated organism is prepared by making a suitable dilution so that a convenient velocity may be obtained. The dilution is best made with frog Ringer's solution containing 0·65 % NaCl, 0·014 % KCl and 0·012 % $CaCl_2$. No $NaHCO_3$ is added as it would cause an increase in p_H due to loss of CO_2. It has been found that, while preparations in which the dilution has been made with water are apt to show some falling off in activity after several hours, those made up with Ringer's solution keep well even at room temperature for 6–8 hours, which is well within the time necessary for one series of experiments to be made. The preparation is kept on ice during the course of the experiments. With regard to the action of the Ringer's solution it is necessary to note that the effect of the added salts on the shape of the curves is very slight indeed. The actual amount of salts present in the aerobic experiments is a third of those present in normal Ringer's solution and in the anaerobic experiments at most a tenth. If a comparison is made of the p_H-activity curve of the methylene blue reduction in the presence of well-washed bacteria in distilled water and bacteria in the presence of a concentration of salts corresponding to Ringer's solution, the velocity is reduced by about 10 % in the latter case, but the curve follows that obtained for the washed preparation. The aerobic oxidations show a very slight difference between the initial rates of respiration, but a difference is noted in the activity after several hours. The activity of the washed preparation has fallen by about 15–20 % after 6 hours. This difference is not nearly so marked with the reduction of methylene blue, so that some alteration in the oxygenase is possibly indicated. The study of the oxidation of succinate is complicated by the fact that after two or three days the organism loses its capacity for the aerobic oxidation at 16°. Fresh preparations of bacteria oxidise succinate at

this temperature and the oxidation at 40° and the methylene blue reduction are unaffected. The technique followed for the aerobic and anaerobic experiments is essentially the same as that described previously.

The buffers used were the Clark-Lubs series of potassium hydrogen phthalate-NaOH, KH_2PO_4-KOH and boric acid-KCl, NaOH. A borax-KH_2PO_4 buffer (Kolthoff) was also employed. This buffer is to be highly recommended as it may be used to check any effects of the phosphate or borate ions *per se* over a considerable portion of their range. The p_H of the reaction mixtures was checked electrometrically at the beginning and end of numerous experiments. The p_H of the undiluted bacterial suspension was in general about 7·4. The bacteria if present in large amounts have a considerable buffering capacity but, as they are used in relatively small amounts in the actual experiments and the solutions of buffer added were always present in a concentration of at least $M/120$, the p_H of the mixture remained practically that of the buffer solutions. The substrate concentration and the total nitrogen of the bacteria present are given in the separate experiments. The substrate concentration taken was always very much greater than that necessary for saturation of the enzyme. The total nitrogen of the bacteria may be used as a measure of the dry weight of bacteria present, as noted previously.

As a test of the aerobic method the following experiment was undertaken. A preparation of bacteria was made by dilution of the stock with Ringer's solution and treated with toluene for exactly half an hour. This time was made the standard time for treatment. The procedure followed was to shake the suspension well with toluene at the beginning of the treatment and to shake at intervals of 5 minutes. The treatment was always made at room temperature. The lower layer of bacteria was then pipetted free from the toluene layer. A small amount of toluene is always present in the material used for experiment and although this does not seem to affect the anaerobic experiments there is a possibility that the presence of the toluene would affect the manometric readings. The same amount of bacterial suspension was therefore placed in both cups of the Barcroft compensating manometer. The suspension thus prepared was diluted to various extents, and the oxidation of lactate at p_H 7·6 and 40° was studied. After 4 hours the stock suspension of the treated organism was again diluted and the experiments repeated. The results are shown on Fig. 1. It will be seen that the velocity of the oxidation is almost proportional to the amount of bacteria. It will also be noted that there is no significant difference between the results obtained half an hour and 4 hours after treatment with toluene.

Anaerobic experiments.

The reduction of methylene blue at 40° in the Thunberg tube in the presence of a standard concentration of substrate and a known amount of bacteria was taken to a standard of 90 % reduction of the methylene blue present. The concentration of this dye used throughout was 1/30,000 for each experiment.

The toluene-treated organism does not reduce methylene blue in the absence of substrate even after several days. A standard consisting of the same amount of bacteria used in the experiments and an amount of dye corresponding to 90 % reduction may be preserved for some time. Fig. 2 represents the oxidation of formate by methylene blue at 40°. The buffer solutions used for obtaining various parts of the curve are indicated on the figure. It will be seen that the curve from p_H 4 to 7 rises steadily. There is an optimum at about

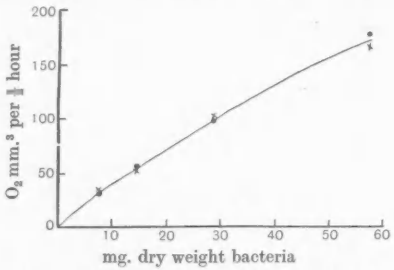
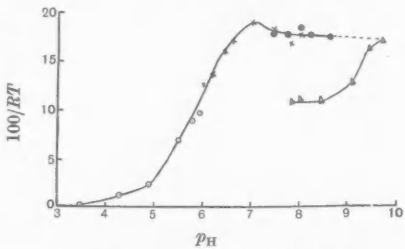


Fig. 1. The aerobic oxidation of $M/60$ lactate at p_H 7·6 and 40°.

Ordinate: oxygen uptake in initial 30 minutes.
Abscissa: dry weight of bacteria in mg.; obtained by taking the total nitrogen as representing 8·3 % of the dry weight of the bacteria.
Values shown by dots represent the velocities half an hour after treatment and those shown by crosses 4 hours after treatment.



⊙–⊙–⊙ Phthalate. ×–×–× Phosphate. ⊗–⊗–⊗ Borax-Phosphate. △–△–△ Borate.

Fig. 2. The anaerobic oxidation of $M/120$ formate by toluene-treated *B. coli* in the presence of 1/30,000 methylene blue at 40°.

Ordinate: velocity, this being the reciprocal of the reduction time for 90 % reduction of the methylene blue × 100.
Abscissa: p_H.
Bacterial nitrogen present for each experiment 0·192 mg.

p_H 7 after which the curve falls slightly, rising again though not to the same level from p_H 7·8 to 9·0. It will also be noted that the velocity in borate buffer at p_H 7·8 is considerably less than in phosphate at this point. In the more alkaline region the curve falls rapidly owing to irreversible inactivation of the enzyme as will be shown later. Fig. 3 shows the anaerobic oxidation of lactate, and it will be seen that the curve is similar in shape to the formate curve, but no peak is to be noted. Fig. 4 represents the anaerobic oxidation of succinate. The curve is of the same shape as that obtained for lactate but

the maximum is not reached until a p_H of 8·6, and the plateau is only of short duration.

The effect of the borate buffer at the same p_H as the phosphate buffer is to be noted, though in a different degree, with all three dehydrogenases. It was at first thought that the difference in velocity at the point where the p_H was the same might be explained by (*a*) an inhibiting effect of the borate ion or (*b*) an accelerating effect of the phosphate ion. But this latter effect is

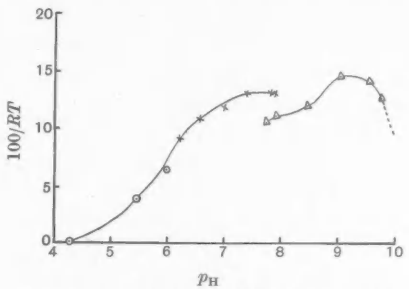


Fig. 3. The oxidation of $M/120$ lactate at 40°.
Ordinate and abscissa as for Fig. 2. Bacterial nitrogen 0·48 mg.

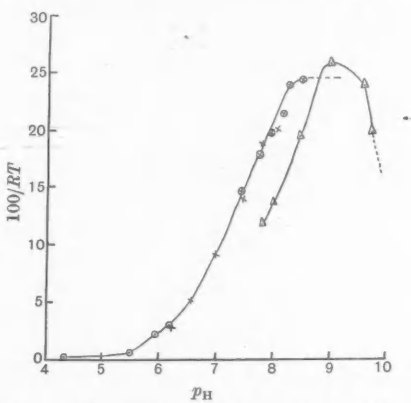


Fig. 4. The oxidation of $M/120$ succinate at 40°.
Bacterial nitrogen 0·96 mg.

rendered unlikely in that no appreciable difference is noted at the change from phosphate to phthalate buffers. Experiments carried out with a borax-phosphate buffer gave a velocity equal to that obtained for the phosphate buffer. The effect of the borate buffer is not due to the presence of the KCl. Experiments carried out with diluted borate buffer and phosphate buffer to which KCl was added did not show any alteration in velocity. It will be noted that the curve obtained with borate rises to give approximately the same

velocity as that for borax-phosphate buffer. The effect of the borate buffer is always most marked at p_H 7·8–8·0. It would perhaps seem that the decrease in velocity was due to an effect of the undissociated boric acid. The p_K of boric acid is 9·24 so that at p_H 7·8 the acid is only slightly dissociated.

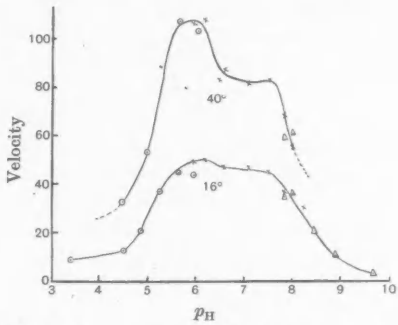
Aerobic oxidations.

The toluene-treated organism has no endogenous oxidation, so that the results obtained represent oxidation of the substrates only. The method employed has been described previously. The Barcroft manometers when set up were swung open to the air for exactly 5 minutes. The taps were then shut and the initial readings taken. Readings were then taken at 15-minute intervals. The velocity was obtained from the difference in the readings after 15 and 30 minutes. A reading at 45 minutes served to show whether the velocity was still constant. At the more acid and alkaline p_H values the velocity fell off indicating enzyme inactivation. At values ranging from p_H 5 to 9 there was no appreciable diminution. The p_H was checked electrometrically at the beginning and end of the experiments. There is no appreciable alteration in the oxidation of lactate and succinate, but with formate there is a change of about 0·2 of a unit with the concentration of buffer used. This change is due to the fact that the formate is oxidised to bicarbonate. This dissociates at p_H 7·4, the CO_2 being taken up by the KOH in the manometer. Thus the effect on p_H of the oxidation of formate is the same as that of adding an equivalent of NaOH. The actual change in p_H during the course of the experiment is probably not more than 0·1 of a unit. The extra difference was probably due to the fact that further oxidation had occurred during the interval between removing the solution from the manometer pot and measuring the p_H. At the time when the velocity was measured, oxidation of 50 % at most of the formate had taken place. This would raise the p_H of $M/20$ buffer solution by about 0·1 of a unit. In general this figure has been added to the initial p_H reading of the aerobic formate experiments.

Figs. 5, 6 and 7 represent the aerobic oxidations of formate, lactate and succinate respectively. It will be seen that the curves obtained for formate and lactate at 16° and 40° show little difference in shape with increase of temperature. It will also be noted that there is practically no difference between the velocities of oxidation where the change is made from phosphate to borate buffer. A similar result was found by Cook and Stephenson [1928] for the aerobic oxidation of lactate by the untreated organism.

Reversibility of the effect of acid and alkaline p_H *on the aerobic and anaerobic systems.*

It is necessary before any attempt can be made to interpret the nature of the curves to observe the action of the more acid and alkaline buffers on the reactions. The curves show that the velocity is considerably diminished at p_H 3 and 10. The procedure adopted was as follows: 50 cc. of the toluene-



⊙–⊙–⊙ Phthalate. ×–×–× Phosphate. △–△–△ Borate.

Fig. 5. The aerobic oxidation of $M/120$ formate in atmospheric oxygen at 16° and 40°.

Ordinate: velocity, being difference between oxygen uptakes in the readings at 15 and 30 minutes.
Abscissa: p_H.
Bacterial nitrogen for each experiment at 40° 0·6 mg.; at 16° 1·2 mg.

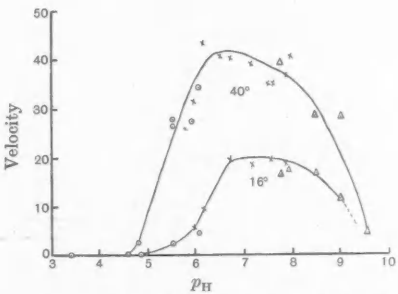


Fig. 6. The oxidation of $M/120$ lactate at 16° and 40°.

Ordinate and abscissa as for Fig. 5. Bacterial nitrogen at both temperatures 1·2 mg.

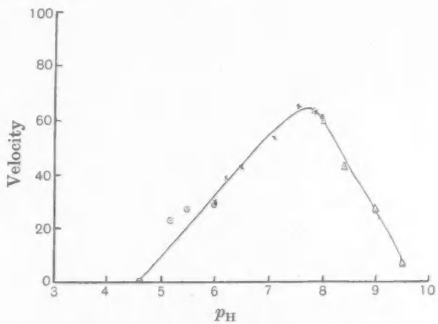


Fig. 7. The oxidation of $M/120$ succinate at 40°.

Bacterial nitrogen 1·2 mg.

treated organism was prepared and divided into 10 cc. samples. To *A* were added 20 cc. of Ringer's solution; to *B* 20 cc. of phthalate buffer at p_H 3·2, the resultant p_H being 3·38; to *C* were added 20 cc. of phosphate buffer at p_H 7·2; to *D* 20 cc. of borate buffer at p_H 10, the resultant p_H being 9·7; *E* the residual 10 cc. served as a second control and was kept on ice. The suspensions were well mixed and allowed to stand at 18° for 1½ hours. The mixtures were then centrifuged and each sample made up to the original volume of 10 cc. The aerobic oxidation of *M*/120 formate at p_H 6·3, and of *M*/120 lactate and succinate at p_H 7·6 and 40° was then investigated. The methylene blue reductions were carried out with the same concentrations of substrates, but at p_H 7·6 in each case. The results are shown in Table I. In these experi-

Table I. *Percentage of original velocity remaining after exposure of the bacteria to the* p_H *shown for 1½ hours at 18°.*

Methylene blue reductions: p_H 7·6.
Oxygen reduction: succinate and lactate, p_H 7·6; formate, p_H 6·3.
Temperature 40°.

	Bacteria exposed to							
	A Ringer		*B* p_H 3·38		*C* p_H 7·2		*D* p_H 9·7	
	O_2	M.b.	O_2	M.b.	O_2	M.b.	O_2	M.b.
Formate	100	100	12·2*	56·5	72·7	100	36	80·5
Succinate	,,	,,	7·0	<3·0	86·3	95·7	58·6	48·3
Lactate	,,	,,	23·6	,,	109	88·1	61·6	74

* Initial velocity about 60 % of original velocity.

ments a reduction of less than 15 % is not to be considered significant, as it is somewhat difficult to ensure the presence of equal amounts of bacteria in the several experiments. The results show that exposure to an acid p_H diminishes considerably the activity of lactic and succinic dehydrogenases, formic still retaining rather more than half its activity. At p_H 7 none of the dehydrogenases is appreciably affected, but at p_H 9·7 the dehydrogenases are affected in the order of activity succinic, lactic and formic. These results agree with those obtained by Quastel and Wooldridge [1927] for the untreated organism. In the aerobic experiments the effect of the acid p_H is most marked with succinate, this being followed by formate and lactate. At p_H 7·2 the oxidation of lactate and succinate is not appreciably affected, but that of formate is decreased by 23 %, while at a more alkaline p_H the effect is in the order formate, succinate, and lactate.

Discussion.

The results obtained in the aerobic oxidations are more complicated in that they involve a second factor in the activation of the oxygen. An interpretation must first be given to the anaerobic oxidations in which it is clear that dehydrogenation of the substrate is the main factor concerned. A glance

at Figs. 2, 3 and 4 will show that the p_H-activity curves of the dehydrogenases are of quite a different type from those obtained with hydrolytic enzymes.

Michaelis and Davidsohn [1911] suggested from the results obtained on the p_H-activity curve of invertase that the enzyme was an amphoteric electrolyte. This conception has been extended by Woolf [1931] who has based his conclusions on the results obtained with fumarase [Mann and Woolf, 1930]. Woolf points out that the symmetrical p_H-activity curve obtained for hydrating enzymes is not given by dehydrogenases, and suggests that the curves represent the action of the enzyme as an ionised acid or an un-ionised base. The curves shown in Figs. 2, 3 and 4 therefore represent the dissociation curve of an acid or the dissociation residue curve of a base. But the curves obtained, especially those in Fig. 2, show deviations from this ideal form. A further study of the formate oxidation is in progress. The curves obtained for lactate and succinate are of the same type as that obtained for formate, although the point at which the curve reaches the plateau is different with the different substrates (Table II).

Table II.

	Formate	Lactate	Succinate
M.b. plateau starts (p_H)	7·0	7·4	8·5
O_2 optimum (p_H)	6·0	6·3	7·6
p_K of acid	3·68	3·86	5·28

The figures obtained for the maximum velocity in oxygen will be referred to later. It will be seen from Table II that there appears to be some relation between the degree of dissociation of the acid concerned and the activity of the dehydrogenase. The p_K of succinic acid has been taken as that of the second ionisation. It may be postulated tentatively that the dehydrogenases are acting as un-ionised bases, the activity being dependent on combination with the fully dissociated acid. This active enzyme-substrate complex then breaks down, the hydrogen of the substrate being transferred to the methylene blue.

The curve obtained for succinate dehydrogenation is similar to that obtained by Ohlsson for extracted muscle succinoxidase. This writer also observed the inhibitory effect of the borate buffer, but continued his experiments in glycine-phosphate buffer. This buffer is not satisfactory for work with bacteria, as deamination may occur. The curve obtained by Quastel and Whetham for resting untreated *B. coli* rises to an optimum at about p_H 7·2 and continues as a plateau to p_H 10. No inhibitory effect is noted with borate buffer. These results may be explained by the fact that the p_H-activity curve of the toluene-treated organism resembles more closely that of an extracted enzyme. It is perhaps significant that the velocity of dehydrogenation is increased by about 30 % after toluene-treatment. In the aerobic experiments with formate and lactate there is no significant difference in the shape of the curves at 16° and 40°. It would therefore appear that when activation of oxygen is the limiting factor, hydrogen ion concentration does not alter the relation between

dehydrogenase and oxygenase. Figs. 5, 6 and 7 show that the curves obtained in some respects resemble those for the dehydrogenases, but that the oxygen optima have been shifted in relation to the edge of the methylene blue plateau to the acid side in all three cases. In Table II the oxygen optima are given and it will be seen that the shift is in each case equal to approximately one p_H unit. It will also be noted from the figures that at the point where a change is made from borate to phosphate buffer no appreciable change in velocity occurs, although there is a difference of about 40 % in velocity at this point in the anaerobic process. A similar effect has been noted by Cook, Haldane and Mapson [1931] with the action of 8-hydroxyquinolinesulphonic acid. From these experiments it is evident that for the aerobic oxidations the activity of the dehydrogenase is not always the limiting factor in the reaction. The shift of the p_H optima in the aerobic oxidations would perhaps indicate that it is the activity of the oxygenase that is limiting the reaction. It will also be seen from Table I that after exposure to p_H 3·38 although there is a considerable diminution in the activity of the lactic and succinic dehydrogenases there is still an appreciable oxygen uptake. With formate the initial velocity corresponds to about 60 % of the original velocity, but the value given represents the velocity when the system is in a steady state.

It is difficult to give any complete interpretation of the curves obtained in the aerobic experiments—as in all cases the process is complicated by the action of the dehydrogenase. A study of the oxidation of indophenol or some related compound in which activation of the oxygen is the sole process concerned would possibly throw some light on this process.

It is evident from these results that the process of oxidation is one of considerable complexity and that the study of the effect of hydrogen ion concentration on the oxidative mechanisms gives us one method of attack on the mechanism of correlation of the various enzymes concerned.

Summary.

1. A study has been made of the effect of hydrogen ion concentration on the aerobic and anaerobic oxidations of formate, lactate and succinate by toluene-treated *B. coli*.

2. The anaerobic oxidations in the presence of methylene blue give p_H-activity curves which rise steadily from the acid region to a plateau which starts at about p_H 7·0 with formate, p_H 7·4 with lactate and p_H 8·5 with succinate. There is a steady drop from the plateau at about p_H 9·5 in all cases.

3. The p_H-activity curves obtained in the aerobic oxidation of the three substrates resemble in general shape the curves for the dehydrogenases, but the optima have been shifted to the acid side.

4. Exposure to solutions of low p_H inactivates the dehydrogenases in the order succinate, lactate and formate. The aerobic mechanism is affected about equally in each instance.

5. The effect is not so marked in the alkaline region. The oxidation of succinate both anaerobically and aerobically and the aerobic oxidation of formate are the mechanisms most affected.

It is with very great pleasure that we express our thanks to Prof. J. B. S. Haldane for many valuable suggestions and ever ready advice. We wish to thank Dr B. Woolf for allowing us to read his paper in proof. To Sir F. G. Hopkins we are indebted for interest and encouragement.

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LXIII. THE RELATIONSHIP BETWEEN THE RESPIRATORY CATALYSTS OF *B. COLI*.

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During the process of respiration in the cell two different types of activation by catalysts have been described. On the one hand organic molecules are rendered more susceptible to oxidation, and on the other oxidising substances more prone to reduction. The first type of activation commonly leads to dehydrogenation in Wieland's sense, and is carried out by dehydrogenases. The activation of oxygen has been particularly stressed by Warburg [1926]. He describes the responsible catalyst as the *Atmungsferment*. As, however, we cannot agree with his view that it is the only catalyst concerned in respiration we prefer to employ the term oxygenase in accordance with the usual practice of naming an enzyme after its substrate. Bach's [1913] oxygenase is possibly not an oxygen activator, but an enzyme which, in conjunction with catechol or a related substance, reduces oxygen to hydrogen peroxide. The main object of the present research was to determine the relationship between these two types of activation. Whereas the dehydrogenations so far studied in living cells have usually been simple, the oxidations by molecular oxygen have almost always been complex. Thus the complete oxidation of glucose must involve very many consecutive reactions, and even that of alcohol or acetic acid more than one. Hence it is difficult to interpret the effects of heat, poisons, or other alteratives on such complex processes. Cook [1930] showed that after treatment with toluene-saturated water *B. coli* still performed the following oxidations:

$$2HOOC{-}CH_2{-}CH_2{-}COOH + O_2 = 2HOOC{-}CH{=}CH{-}COOH + 2H_2O$$

$$2CH_3{-}\underset{\underset{H}{|}}{\overset{\overset{O}{\|}}{C}}{-}COOH + O_2 = 2CH_3{-}CO{-}COOH + 2H_2O$$

$$2H{-}COOH + O_2 = 2CO_2 + 2H_2O$$

The oxidation of formic acid proceeds to 98 % completion, those of succinic to fumaric, and of lactic to pyruvic acids proceed to about 82 % and 76 % respectively. In neither case does the oxidation proceed any further, no CO_2

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being produced in the latter two. The rates do not differ greatly from those in the untreated organism.

The toluene-treated organism has the further advantage that it has no metabolism in the absence of added substrate. Not only does it not consume O_2 but it does not reduce methylene blue *in vacuo* even after several days.

The bacteria used were a strain (N.T.C.) from the National Type Culture collection, and a strain (O.P.S.) from the University Pathological Department, suspensions being made as described by Cook and Stephenson [1928], with frog Ringer's solution. These were diluted with water so as to give a suitable rate of reduction of oxygen or methylene blue. Fuller details are given by Cook and Alcock [1931]. Carbon monoxide was obtained from a cylinder. It contained small amounts of nitrogen and hydrogen as impurities, as well as CO_2, which was absorbed in consequence of the technique used.

Observations on the dehydrogenases of the toluene-treated organism.

The properties of succinic, lactic, and formic dehydrogenases have been studied by Quastel and his colleagues from 1924 onwards and by Cook [1930], but certain further observations were required. The Thunberg vacuum tubes used contained 2 cc. 0·2 *M* phosphate buffer, 1 cc. 0·002 *M* methylene blue, 0·5 cc. 0·2 *M* substrate, and 1·5 cc. H_2O, to which inhibitors were added as required. These were brought to the required p_H with H_3PO_4 or NaOH.

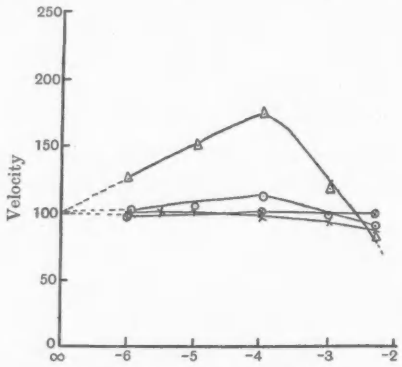
Finally, 1 cc. of bacterial suspension diluted with water was added. The substrate concentration was thus *M*/120. The tubes were evacuated for 1 minute and then placed in a bath at 40°. The time taken for 90 % reduction was measured. All measurements were made at least in duplicate.

Effects of cyanide.

The effects of KCN on the rate of methylene blue reduction are shown in Fig. 1. The ordinates are the reciprocals of the reduction times. It will be seen that there is a moderate activation by low concentrations of cyanide, which is clearly significant in the case of succinic dehydrogenase. The greater effect of cyanide on formic dehydrogenase at the more acid p_H is doubtless due to the greater amount of HCN in equilibrium with cyanide ions at that p_H. The irregularities are perhaps due to the fact that variable amounts of HCN were pumped off in different experiments. It will be seen that there is no very serious inactivation of any of the dehydrogenases below a concentration of 0·002 *M*, whereas catalase and peroxidase are largely inactivated by concentrations of 10^{-5} *M*. Hence any marked effects of cyanide concentrations below 0·002 *M*, such as are shown in Fig. 3 are not due to effects on the dehydrogenases. No reduction of methylene blue occurs in presence of cyanide and buffer alone.

Effects of carbon monoxide.

In order to study the effect of CO this gas was passed through alkaline pyrogallol solutions to absorb O_2 and CO_2. The substrate, dissolved in only 0·1 cc. of water, was placed in the small limb of the vacuum tube, which was evacuated; CO was run in, the tube re-evacuated and more CO added. It was then cooled on ice, shaken vigorously for exactly 3 minutes, and placed in a thermostat. Controls were filled in just the same way with N_2. CO has no



△–△–△ Succinate p_H 76. ⊙–⊙–⊙ Lactate p_H 76.
⊗–⊗–⊗ Formate p_H 76. ×–×–× Formate p_H 63.

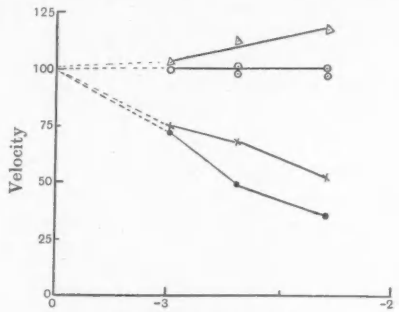
Fig. 1. Effect of KCN on rates of methylene blue reduction.

effect on lactic dehydrogenase at 40°. At 25° it seems to have a small effect on formic dehydrogenase. Thus at 25° the reduction times in N_2 were 4·15, 4·25, 4·25, in CO 5·15, 5·45. However, at 16° there is no such effect. Thus reduction times in N_2 were 17·00, 17·10 and 16·45 minutes, in CO 16·40, 16·55 and 16·50. As the experiments on oxygen uptake in presence of CO were almost all made at this temperature, it is clear that the dehydrogenases were not being poisoned in them.

The Hecht-Eichholtz reagents and phenylurethane.

Hecht and Eichholtz [1929] and Eichholtz [1929] introduced the use of a system of organic compounds as heavy metal reagents in biochemical research. They form complexes with heavy metals, and the inhibition or acceleration [Krah, 1930] of a reaction is taken to show that the catalyst concerned contains a metal which combines with the reagent added. We have used the reagents 8-hydroxyquinolinesulphonic acid (Q), 2-aminophenol-4-sulphonic acid (A), and 1-amino-8-napthol-4-sulphonic acid (N) at 40°. A has no marked effect on any of the dehydrogenases. Q has no effect on reduction by lactates. In very high concentrations such as 0·4 % Q slightly accelerates

reduction by succinate, the velocity being increased by 21 %. Q has a large effect on formate, as shown in Fig. 2. The effect is greater at p_H 6·3 than at 7·6, as with the acidic poisons used by Myrbäck [1926]. N has a very great inhibitory effect on reduction by formate. These results suggest that formic dehydrogenase contains copper and not iron. For Q and N, which inhibit, both



△–△–△ Succinate p_H 7·6. ⊙–⊙–⊙ Lactate p_H 7·6.
×–×–× Formate p_H 7·6. •–•–• Formate p_H 6·3.

Fig. 2. Effect of 8-hydroxyquinolinesulphonic acid on rates of methylene blue reduction.

unite with copper. Q also unites with Mn, Co, and Ni, N also with Fe, while A unites with Fe only. Inhibition by Q is independent of variations in the concentration of methylene blue, and is no greater when the bacteria have stood with the reagent for 1½ hours before the methylene blue is added.

No inhibition was obtained with phenylurethane on lactic or formic dehydrogenases.

Experiments on O_2 reduction.

The Method

Oxygen uptakes were studied in Barcroft apparatus. Each cup contained 1·0 cc. of a suspension of the toluene-treated organism, 1·0 cc. of a 0·5 *M* buffer solution, 0·5 cc. of a donator solution for which distilled water was substituted in the left-hand pot, and 0·5 cc. of distilled water or solution of an inhibitor. The p_H was always 7·6 in the case of succinate and lactate, this hydrogen ion concentration being nearly optimal, as shown by Cook and Alcock [1931]. In the case of formate, the p_H was either 7·6 or more usually the optimal 6·3. From time to time the p_H after the end of the reaction was measured with a hydrogen electrode and was found to be unaltered in the case of lactate and succinate. Formate solutions became more alkaline by about 0·2 p_H at most. The substrate concentration was usually *M*/60, as at this concentration the enzymes concerned are saturated with their substrates, and the rate of oxidation does not therefore fall off with a moderate diminution in the substrate concentration. Gas mixtures were made up and stored over water in aspirators. They were analysed with the Haldane apparatus.

To introduce them into the Barcroft apparatus the right-hand tube of the latter is connected to the aspirator with rubber tubing. The apparatus is then brought nearly into thermal equilibrium with the thermostat. The left-hand pot is left in position, the right-hand pot held so that a small crevice is left between it and its stopper. The gas is then introduced, the pot being held so close that there is always a positive pressure within it, as shown by the manometer. About half a litre is then run through, the pot pushed home and the tap closed. After 5 minutes' shaking in the bath the taps are opened to level the oil in the manometer, and the experiment begins. Analyses of gas from the pot agreed with that introduced to within 0·3 %. In a few experiments the manometers were evacuated, and both cups filled with gas mixture. On repeating the process several times the air is pretty completely replaced by gas mixture. The small central pot contained a roll of filter paper soaked in KOH to absorb CO_2 [Dixon and Elliott, 1930]. The apparent rates of oxygen uptake often did not become steady until half an hour had elapsed, due in part to the gases going into solution (see Fig. 5). The data here given refer to the linear parts of the curves. All experiments, including controls, were done at least in duplicate.

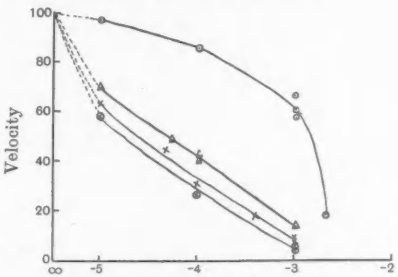
In the case of formate and lactate the oxygen uptake remained fairly constant from day to day. There were, however, occasional fluctuations, generally decreases in oxidising power on keeping. Hence we have not given full weight to experiments in which the effect of a poison was not measured simultaneously with a normal control, although when controls were done on the same day the error is likely to be small. In the case of succinate the oxidising power often fell off in the first few days of keeping a bacterial suspension. The power of reducing methylene blue sometimes fell off simultaneously; but often it remained normal when the oxygen uptake had dropped to less than 10 % of the original value. Hence our data on succinate oxidation are rather incomplete.

Effect of cyanide on oxygen uptake.

Precise measurements of the effect of cyanide on oxygen uptake are difficult, because the velocity of uptake, when inhibition is considerable, sometimes tends to fall off slightly with time. On the other hand, there is no question whatever as to the difference in the susceptibility of lactic dehydrogenase on the one hand, and succinic and formic on the other. This is at once clear from Fig. 3. The difference between the latter two is not so clearly significant, and may be due to the stimulating effect of cyanide on succinic dehydrogenase. As the dehydrogenases are hardly inhibited in concentrations below 10^{-3} M it is clear that the main effect here studied is a direct effect either on the oxygenase or on some intermediate substance. The dissociation constant of the oxygenase-cyanide compound (if we regard the effect as due to combination) would seem to be about $1{\cdot}5 \times 10^{-3}$ M in the case of lactate, 5×10^{-5} M in that of succinate, and 2×10^{-5} M in that of formate.

Effect of phenylurethane on oxygen uptake.

Phenylurethane had a slight inhibitory effect on oxygen reduction by lactate and formate at 16°. The former process was reduced to 78 % of its original velocity by 0·5 cc. saturated phenylurethane solution in the total volume of 3 cc. Formate oxidation was reduced to 75 % by saturated phenylurethane solution and to 83 % by $M/6000$ solution.



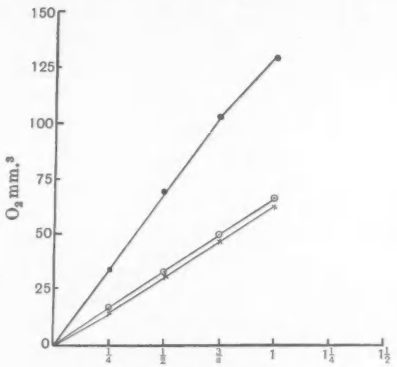
⊙–⊙–⊙ Lactate. △–△–△ Succinate.
×–×–× Formate p_H 7·6. ⊗–⊗–⊗ Formate p_H 6·3.

Fig. 3. Effect of KCN on rates of O_2 reduction.

When, however, the oxidation had already been partly inhibited by cyanide, saturated phenylurethane was more effective. The uptake of lactate in presence of 10^{-3} M KCN was reduced to 24 % of its value with KCN alone, that of formate in presence of 10^{-5} M KCN to 56 % of its former value.

Effect of carbon monoxide.

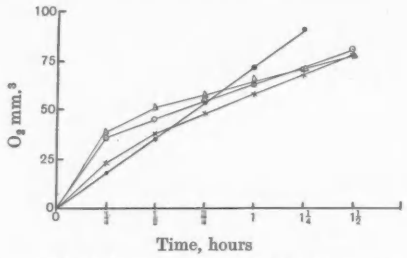
A large number of experiments have been done in mixtures containing varying amounts of CO, O_2 and N_2. As a preliminary it was shown that the



•–•–• Air. ⊙–⊙–⊙ 24·5 % CO, 5·6 % O_2. ×–×–× 49·2 % CO, 9·5 % O_2.

Fig. 4. Oxygen uptakes at 16° and p_H 6·3 in presence of formate from air and two mixtures containing different amounts of CO, but similar CO/O_2 ratios.

oxygen uptake remains unaltered when air is replaced by pure O_2, or diluted with N_2 so that the partial pressure of O_2 is reduced to 5 %. Hence neither diffusion of O_2 nor incomplete saturation of oxygenase with it was a limiting factor. Several experiments, of which Fig. 4 is typical, showed that as found by Warburg [1926] the inhibition by CO depends solely on the ratio of CO to O_2, and not on the partial pressure of CO. It at once becomes clear that the oxidation of formate is more sensitive to CO than that of succinate or lactate. If V be the velocity of O_2 uptake in absence of CO, v that in its



•–•–• Air. ×–×–× CO/O_2 4·1. ⊙–⊙–⊙ CO/O_2 7·1. △–△–△ CO/O_2 13·6.

Fig. 5. Oxygen uptakes at 16° and p_H 7·6 in presence of succinate from air and various gas mixtures containing CO.

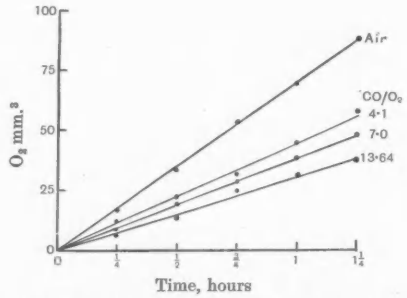


Fig. 5 *a*. Data of Fig. 5 plotted after the first half-hour.

presence, r the ratio of partial pressures of CO to O_2, and K the affinity of oxygenase for oxygen divided by its affinity for CO, then Warburg shows that $v = \frac{KV}{K+r}$, or $K = \frac{r}{\frac{V}{v} - 1}$, provided certain assumptions examined later are made.

From such data as those of Figs. 5, 6 and 7 it is possible to calculate values of K. Unfortunately the majority of our data are dubious for one of four reasons. Duplicates do not agree, uptakes are not linear, experiments with and without CO were not done simultaneously, or the temperature had varied. The results vitiated in any of these ways are grouped as second-class results in Table I. No results whatever have been rejected.

Details of a typical experiment are given below.

Two manometers containing N.T.C. bacilli and $M/60$ formate at 16° and p_H 6·3 gave uptakes of 76·6 and 78·9 mm.³ O_2 in the half-hour between ½ an hour and 1 hour after the beginning of the experiment. Two others containing the above in presence of 59·9 % CO and 7·49 % O_2 gave uptakes of 56·6 and 60·8 mm.³ O_2 in the 2 hours from ¾ hour to 2¾ hour after the beginning. In each case the O_2 uptake was linear over this period. The mean uptakes per hour were therefore 155·5 mm.³, and 29·34 mm.³ The velocity was thus reduced to 18·9 %, giving a K value of 1·86, with an uncertainty of +0·11, allowing for the differences between duplicate oxygen uptakes and CO estimations.

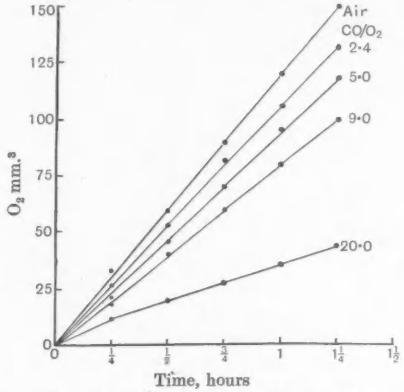


Fig. 6. Oxygen uptakes at 16° and p_H 7·6 in presence of lactate from air and various mixtures containing CO.

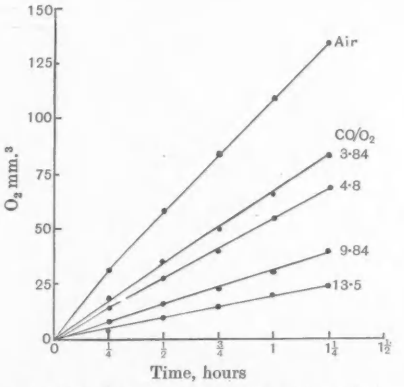


Fig. 7. Oxygen uptakes at 16° and p_H 6·3 in presence of formate from air and various gas mixtures containing CO (O.P.S. bacilli).

On the other hand, in some of the second-class experiments duplicates disagreed very markedly. Thus in the lactate experiment giving a K value of 17·4 the duplicate CO uptakes were 21·1 and 27·7 mm.³ The lower value, which was probably correct, would have given $K = 11·1$.

It will be seen that the two strains used differed considerably as regards the sensitivity of formate oxidation to CO. There were no significant differences as regards succinate and lactate oxidation. The difference between the two strains possibly arose during the experiments. Two second-class experiments on the N.T.C. strain in July 1930 gave values of 4·15 and 3·20 for K, while the other experiments at p_H 6·3 from October onwards gave values of 2·35 or lower. There can be no serious doubts as to the significance of the differences of the K values found. It will be observed that there is no overlapping among the first-class values. The second-class values do not show any certain difference between succinate and lactate, but both these differ clearly from formate.

Table I.

Values of K (ratio of CO to O_2 giving 50 % inhibition) at 16°.

Substrate and race	First-class results	Median	Mean	Second-class results	Median
Succinate	5·9, 6·8	6·3	6·3	5·0, 5·0, 6·0, 10·3, 13·8, 16·5	8·2
Lactate	8·2, 9·5, 9·6, 9·7, 11·8, 12·2, 15·7*	9·7	10·1	3·8, 6·5, 6·9, 7·4, 7·8, 8·7, 10·2, 11·9, 13·4, 14·4, 16·4, 17·4	9·5
Formate (O.P.S.)	3·39, 3·45, 3·54, 4·14	3·49	3·62	1·75, 3·14, 3·45, 3·88, 4·46	3·45
Formate (N.T.C.)	1·86, 2·27, 2·35, 1·40†	2·27	2·15	0·94, 1·07*, 1·29, 1·67, 2·30, 2·95‡, 3·20, 4·15	1·98

* At 18°. † At p_H 6·0. ‡ At p_H 7·6.

In Fig. 8 the observed velocities for lactate oxidation in presence of CO, as percentages of those in its absence, are plotted against the CO/O_2 ratio.

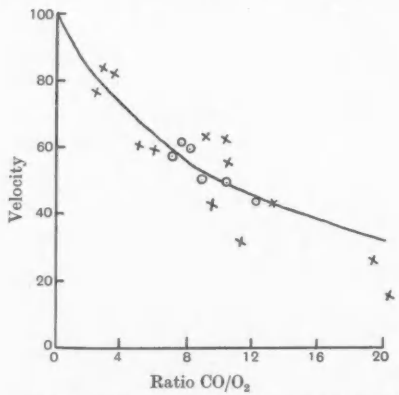


Fig. 8. Velocities of oxygen uptake (as percentages of that in air) at 16° and p_H 7·6 in presence of lactate from gas mixtures containing CO and O_2 in different ratios. The curve represents velocities calculated for $K = 10$.

There is no decided evidence for a significant departure from a rectangular hyperbola, such as Warburg [1926] found in the case of yeast. The two velocities plotted on the right in Fig. 8 are certainly low, corresponding to low

values of K, but neither is reliable. On the other hand, in the case of formate oxidation by O.P.S. bacilli the two lowest velocities, obtained with CO/O_2 ratios of 13·6 and 13·9, give a first-class K of 3·39, and a second-class K of 3·20 respectively. Both the velocities lay between 18 % and 19 % of that in air.

At 40° the effect of CO was much less (Fig. 9). With formate we obtained a first-class value of 9·8, with the O.P.S. strain, and second-class values of

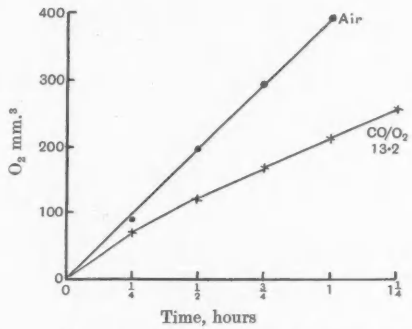


Fig. 9. Oxygen uptakes at 40° and p_H 6·3 in presence of formate from air and a gas mixture containing CO (O.P.S. bacilli).

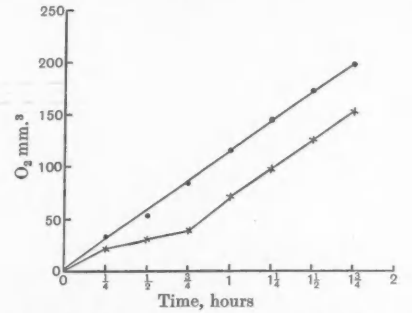


Fig. 10. Oxygen uptakes at 16° and p_H 7·40 in presence of formate from a gas mixture containing 65 % CO and 7·7 % O_2. After 45 minutes this was replaced by air.

40, 9·7, 6·7, and 5·7 with the N.T.C. strain. The effect on lactate oxidation was negligible with a CO/O_2 ratio of 13·2, which would have given 59 % inhibition at 16°. The value of K is probably at least 50.

Inhibition by CO is completely reversible (Fig. 10). Experiments carried out on bacteria exposed to CO in the strongest light at our disposal appeared to show an acceleration of about 5 % as compared with nearly complete darkness. The effect was, however, too small to measure accurately.

Combined effect of cyanide and carbon monoxide.

Four experiments were carried out to see what further inhibition was produced by CO on oxygen uptake already partly inhibited by cyanide. In two second-class experiments with lactate in presence of 0·001 *M* KCN we obtained values of 9·3 and 14·4 for *K*. With formate in presence of 0·001 *M* KCN we obtained, with N.T.C. bacilli, a first-class value of 2·16, and a second-class value of 2·47. Thus in presence of cyanide concentrations which themselves produce an inhibition of 37 % and 70 %, the value of *K* was, if anything, slightly increased, *i.e.* the system rendered less sensitive to CO.

Combined effect of oxalate and carbon monoxide.

As a preliminary to the use of oxalate it was shown that the values of *K* are unaltered by washing the bacilli free from calcium.

Oxalate inhibits the reduction of methylene blue by lactate, and has a similar effect on oxygen reduction. The latter is not, however, quite so great as the former. Thus in one series of experiments performed simultaneously the velocity of methylene blue reduction was reduced to $39{\cdot}7 \pm 2{\cdot}3$ %, that of oxygen reduction to $52{\cdot}6 \pm 1{\cdot}9$ %, the errors being the extreme values calculable from the observations.

Oxalate has no marked effect on O_2 reduction by formate [Cook, 1930] nor on the *K* value of this process. With N.T.C. bacilli we obtained a first-class value of 2·30 and a second-class value of 1·29 in 0·0027 *M* oxalate, in 0·0017 *M* oxalate a second-class value of 1·63. In 0·0076 *M* oxalate a first-class experiment gave $K = 1{\cdot}86$. Thus, if anything, oxalate makes formate oxidation more sensitive to CO, but the effect, if any, is within the limits of experimental error.

On the other hand, oxalate renders the oxidation of lactic acid far less sensitive to CO. In a concentration of *M*/3000 we obtained a first-class value of *K* of 19·0, and second-class values of 42 and 468, which at least demonstrate that there was hardly any inhibition.

The combined effect of phenylurethane and carbon monoxide.

Although, as pointed out above, phenylurethane has only a slight effect on oxygen reduction, it renders the system extremely sensitive to CO. Thus the value of *K* for lactate oxidation in presence of 0·00011 *M* phenylurethane fell to 0·86 in one first-class experiment, that for formate oxidation by N.T.C. bacilli fell to 0·24 and 0·17 in two good second-class experiments. Clearly the ratio of the affinities of the oxygenase for CO and O_2 has been increased about tenfold. It will be remembered that phenylurethane also renders the system more sensitive to cyanide, though in this case the effect is less striking.

The effect of 8-hydroxyquinolinesulphonic acid.

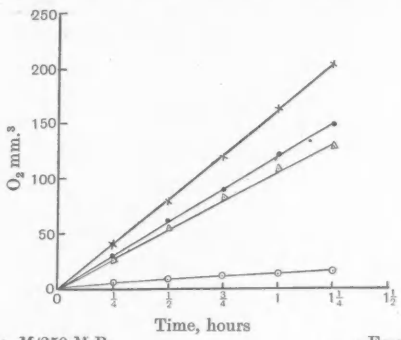
In spite of its marked inhibitory effect on methylene blue reduction by formate, 8-hydroxyquinolinesulphonic acid has no effect on oxygen reduction by this substrate. Thus a 0·004 *M* concentration at 16° gave a 2 % reduction in oxygen uptake by formate, and a 1 % increase in that by lactate, both well within the limits of experimental errors. Nor is there any definite effect on the value of *K* in the case of formate. A second-class experiment on N.T.C. bacilli gave $K = 1{\cdot}68$.

Experiments with mixtures of formate and lactate.

Cook [1930] showed that at 40° the oxygen uptake in presence of lactate and formate is equal to the sum of those in presence of either alone. This was found to be true at 16° also. Thus a suspension absorbed 64·3 mm.³ of O_2 in half an hour in presence of formate, 39·2 in presence of lactate, while in a mixture the quantity was 103·2 mm.³, the calculated value being 103·5. This mixture was now exposed to CO and gave a value of *K* of 1·66. The data are of the second-class, owing to disagreement of duplicates in presence of CO, and the true value was probably higher.

Oxygen uptakes in presence of methylene blue.

A series of experiments was carried out to see how far oxygenase can be replaced by methylene blue. Oxygen uptakes were measured alone, in presence of added methylene blue, of added cyanide and of methylene blue and cyanide. Fig. 11 shows that in the case of formate when the oxygenase has



×–×–× Formate + *M*/250 M.B. •–•–• Formate control.
△–△–△ Formate + *M*/250 M.B. + *M*/1000 KCN. ⊙–⊙–⊙ Formate + *M*/1000 KCN.

Fig. 11. Oxygen uptakes from air at 16° and p_H 6·3 in presence of formate alone, with the addition of KCN, of methylene blue and of both.

been mainly poisoned by cyanide, it can be replaced to a considerable extent by methylene blue. This is reduced by the formic dehydrogenase and re-oxidised by air, this re-oxidation not being prevented by the cyanide. Fleisch

[1924] and Stephenson [1928] have obtained similar results. In other experiments the uptake in presence of formate has been actually increased by the addition of KCN and methylene blue. Thus at p_H 7·6 the oxygen consumption in the same period of half an hour was 47·0 mm.^{3} in the absence of cyanide and methylene blue, and 63·6 when both were present. Duplicates agreed well.

The oxidation of lactate was affected differently. We were never able to get more than 67 % of the original oxidation on adding methylene blue and cyanide, and methylene blue alone reduced the rate of uptake to about 74 % of the normal value. We have no satisfactory results in the case of succinate at 16°. At 40° methylene blue causes a slight acceleration.

The system in which, in presence of cyanide, oxygenase is replaced by methylene blue can be used as a model for the action of CO on the oxygenase. When such a system is exposed to air containing CO the rate of oxygen uptake falls off rather slowly, and finally may settle down to a definite velocity. In the steady state the methylene blue can be seen to be mainly reduced. This is explained by the discovery of Reid [1930] that the re-oxidation of leucomethylene blue is a metal catalysis inhibited by CO. Presumably the KCN and CO between them unite with almost all the catalytically active metal. The degree of inhibition depends on the partial pressure of O_2 as well as that of CO. Thus in presence of formate at p_H 6·3 we obtained an uptake of 11·4 mm.^{3} per hour in presence of 52·5 % CO and 9·68 % O_2, but of 50·8 mm.^{3} in presence of 52·5 % CO and 46·3 % O_2. It had previously been shown that, in the absence of CO, the rate of oxygen uptake did not depend on the partial pressure of O_2. In another set of four experiments the rate was shown to be the same when the ratio of CO to O_2 was kept constant, the absolute amount being altered. The value of K was in one case 0·18, but it varied in different experiments, probably because the amount of catalytically active heavy metal was not constant.

Discussion.

The experiments on methylene blue reduction can be interpreted without difficulty. The stimulating effect of small amounts of cyanide can probably be explained by its combination with small amounts of heavy metal which inhibit succinic dehydrogenase, as shown by Quastel and Wooldridge [1927]. A similar explanation of their activating effect on proteinases has been given by Krebs [1930]. The inhibition by relatively high concentrations of cyanide may be due to its union with heavy metals forming part of the enzymes. But the Hecht-Eichholz reagents give no indication of metal in succinic and lactic dehydrogenases, though they point strongly to the presence of copper in formic dehydrogenase and suggest that copper is inhibiting succinic dehydrogenase.

Cook [1930] showed, by the specific effect of malonate on the oxidation of succinate by methylene blue and oxygen, that the same catalyst was probably activating the succinate in each case, *i.e.* that succinate is activated by succinodehydrogenase before it reduces oxygen. The same is true for lactate. But

this does not hold for formate, as 8-hydroxyquinolinesulphonic acid inhibits the reduction of methylene blue, but not of oxygen. And whereas methylene blue does not fully replace oxygenase in the oxidation of lactate, it more than does so in the case of formate. The suggestion is obvious either that oxygenase is the limiting factor throughout, or that the formate-activating mechanism which is inhibited by 8-hydroxyquinolinesulphonic acid is not normally concerned in oxygen reduction, but is made available for this process by the addition of methylene blue. The work of Stephenson and Stickland [1931] suggests that it may be related to hydrogenase in the same way as lactic dehydrogenase is related to oxygenase. The complexity of the curve relating oxygen reduction by formate to p_H [Cook and Alcock, 1931] suggests that the system concerned is particularly complex.

The fact that CO inhibits O_2 reduction in a competitive manner makes it quite clear that it acts on an activator of O_2, as Warburg has pointed out. On the other hand, the difference between the apparent relative affinities of oxygenase in the three different oxidations studied suggests strongly that we are dealing with three oxygenases which differ from one another in this respect as do the haemoglobins of different species and individuals [Douglas, Haldane and Haldane, 1912; Anson *et al.*, 1924]. Further the relative affinities of the oxygenases concerned in formate oxidation in two different races of *B. coli* differ in this manner. Evidence for the existence of two oxygenases in *Galleria mellonella* which differ in their relative affinities has already been given by Haldane [1927].

It is clear, moreover, that, even if we do not accept the above theory, the actual molecules of oxygenase concerned in formate and lactate oxidation are different. For not only are the oxygen uptakes additive on mixing under normal conditions, but this is still true when they have been reduced by cyanide, so that oxygen activation is clearly the limiting factor. Thus in presence of 10^{-4} *M* KCN the oxygen uptake by formate was 70 mm.^{3} per hour, by lactate 80, by the two together 150. It therefore seems to be impossible that the three dehydrogenases should draw on the same common stock of oxygenase, even if the oxygenase associated with each is of the same molecular species.

Warburg [1926] believes that differences in the apparent affinity ratio K can be reconciled with the existence of only one oxygenase, on the following theory, re-stated in the terminology of Briggs and Haldane [1925] and Wurmser [1930]. Consider the reactions:

$$\underset{e-p-q}{W} + \underset{x}{O_2} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{p}{WO_2} \xrightarrow[\epsilon k_3]{} W + 2O,$$

$$\underset{e-p-q}{W} + \underset{y}{CO} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \underset{q}{WCO}.$$

Where W represents oxygenase, and the molecular concentrations of the reactants and the velocity constants are as above. ϵk_3 is the constant of the

reaction actually measured and is supposed to be independent of the concentration of reducing substrate when this reaches a certain value, but to fall off when this is lowered, as in sugar-free buffer solution, ϵ being reduced from unity to a fraction.

Then the velocity of oxygen uptake is $\epsilon k_3 p$, or

$$\frac{\epsilon k_3 ex}{x+\frac{(k_2+\epsilon k_3)}{k_1}\left(1+\frac{k_4 y}{k_5}\right)}.$$

But x is very large compared with $\frac{k_2+\epsilon k_3}{k_1}$, since if $y=0$, the velocity is independent of x; hence the velocity is

$$\frac{\epsilon k_3 e}{1+\frac{(k_2+\epsilon k_3)k_4 y}{k_1 k_5 x}}.$$

Hence $K=\frac{k_1 k_5}{(k_2+\epsilon k_3)k_4}$, and therefore lies between $\frac{k_1 k_5}{(k_2+k_3)k_4}$ and $\frac{k_1 k_5}{k_2 k_4}$.

If k_2 is small compared with k_3, K may become very large when ϵ is small. The fact that the value of K for lactate oxidation rose when the lactic dehydrogenase was inhibited by oxalate completely confirms Warburg's theory. But it is also clear that the value of K can be altered by other means. For phenylurethane does not greatly reduce the velocity of oxygen uptake in the absence of CO, and hence cannot greatly change ϵ. But yet it has a very large effect in lowering the value of K.

On the theory that there is only one oxygenase, or *Atmungsferment*, we should have to suppose that whereas most of the oxygenase concerned in formate oxidation is in use, only a fraction is active in the case of succinate, and a still smaller fraction in that of lactate. In this case, however, KCN, by inhibiting part of the oxygenase, should diminish the value of K. It does not. The order of susceptibilities to KCN is the same as that to CO, but in this case the apparent affinities are nearly the same for formate and succinate oxidation, both being very much greater than for lactate oxidation. In the case of CO formate is far more susceptible than succinate or lactate. Moreover, whereas the oxygenases associated with lactate and formate oxidation are very stable, that concerned with succinate is unstable, even when the dehydrogenase is unaltered. Again it is known that the oxygenases of different organisms differ widely in the susceptibilities of their CO compounds to light [Keilin, 1929] and that this difference does not run parallel with their apparent affinities to CO, as it should if ϵ is the only variable concerned. Hence without ruling out the possibility of an explanation on Warburg's lines, we regard it as probable that there are at least three different kinds of oxygenase in *B. coli*. They probably all possess the same prosthetic group, but differ, as do haemoglobins, in the molecule to which it is attached.

We find that the inhibition by CO falls off very strongly with temperature. Warburg [1926] found no definite temperature effect, and Keilin [1929] found that in the case of yeast a rise of temperature increased the inhibitory effect of CO on oxygen consumption while having no effect on CO inhibition of

indophenol oxidase. On the other hand, the inhibition by CO of oxygen uptake by heart muscle and potato catechol oxidases showed a falling off with rising temperature, as in our case. We believe that this is due to a real diminution in the ratio of the affinities for CO and O_2, because there is a strong reason to think that oxygen activation is a limiting factor at 40°, even if it is not so at 16° [Cook, 1930]. Thus the majority of cases show a rise of K with temperature. The corresponding quantity for a human haemoglobin rises from 0·0025 at 15° to 0·004 at 37° [Anson *et al.*, 1924]. (Warburg's K is the reciprocal of Barcroft's.)

Finally we have to consider the relation between a dehydrogenase and the oxygenase associated with it. Three possibilities are open. Intermediate "carriers," such as cytochrome, glutathione, or hexuronic acid may diffuse from one to the other more or less freely. One or more molecules of each may be associated in a very small section of the cell, a minute reaction vessel, so to speak. Or the dehydrogenase and oxygenase molecules may be actually in contact. Our results are strongly against the first hypothesis, for we have shown that there is not a common stock of oxygenase, and on the whole in favour of the third. If it is true we can expect both oxygenase and dehydrogenase activity to be limiting factors simultaneously, as, for example, p_H and substrate concentration are in the case of a simple enzyme. Otherwise it is difficult to see why at 16° both CO and oxalate should give approximately hyperbolic curves when inhibiting lactate oxidation. If there were, for example, an excess of oxygenase, we should expect to get no effect from CO until a large amount of the oxygenase was poisoned. But we obtained 25·6 % inhibition of lactate oxidation with a ratio of CO to O_2 of only 2·4, the calculated inhibition being 19 %. For this reason an actual juxtaposition of the two catalysts seems plausible, though far from certain. Case [1931] comes to a similar conclusion regarding two muscle enzymes.

The results obtained must not be regarded as a picture of the mechanism of respiration in a typical cell. *B. coli* is a facultative anaerobe, its dehydrogenases are almost if not quite confined to its surface, and Dr Keilin kindly allows us to state that he finds it to contain cytochrome B and protohaematin, but has been unable to detect cytochrome A or C. A more normal type of cell might behave quite differently.

We have to thank Sir F. G. Hopkins for his great interest in our work, and Mr Meldrum for a valuable criticism.

Summary.

1. We have studied the oxidation of succinate to fumarate, lactate to pyruvate, and formate to bicarbonate by the toluene-treated *Bacillus coli*. These reactions can each be studied uncomplicated by others.

2. Oxidation by methylene blue is little affected by KCN or CO, but the effects of other poisons suggest that formic dehydrogenase contains copper.

3. Oxidation by O_2 is inhibited by KCN and CO. The activators of oxygen concerned in the three catalyses differ in their susceptibilities to these poisons, and are regarded as differing as do different haemoglobins.

4. Phenylurethane has little direct action on these catalysts, but makes them more sensitive to CO and KCN.

5. Conclusions are drawn as to the way in which the various catalysts which are concerned in respiration co-operate with one another.

6. In presence of cyanide methylene blue can replace the activators of oxygen. Like theirs, its action is inhibited by CO.

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LXIV. CASEINOGEN FOR VITAMIN TESTS.

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The basal diet used in vitamin A testing should, when supplemented by cod-liver oil, allow growth to maturity at what may be considered a normal rate; and the mean growth response of groups of animals should be graded to the dose of cod-liver oil given. If growth is restricted by the lack of some factor other than vitamin A, the result, at the best, may show such small differences in response to different doses of cod-liver oil as to make the significance of the differences doubtful. At the worst, the results may be so very irregular that no conclusion whatever can be drawn from them. It has recently been shown [Coward, Key, Dyer and Morgan, 1930] that the kind of caseinogen used in the basal diet for vitamin A testing is of the greatest importance in this respect. One sample of "vitamin-free casein," failed completely to fulfil the required conditions. Some of the rats grew well but the mean responses of groups of rats to different doses of cod-liver oil were slightly graded to the lower doses only and, at a comparatively low level, reached a limit which was far below any "normal" rate. The caseinogen was suspected of being the limiting factor and a fresh sample was used. "Light white casein" bought from the British Drug Houses Ltd. was extracted cold once with alcohol and four times with ether (24 hours each extraction), and the whole experiment repeated with satisfactory results. On the higher doses of cod-liver oil, the growth response of the groups might be considered almost "normal." It was probably as great as could ever be obtained immediately after the cessation of growth due to a deficiency of vitamin A. Moreover, the differences in response of different groups of rats to doses differing by 100 % were great enough to be considered significant even when the possible error of the estimation was allowed at 2ϵ where $\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$. This held through a range of doses from 0·25 mg. to 7·5 mg.

The extraction of the caseinogen was, however, both costly and laborious and it occurred to us that the greater growth-promoting power of "light white casein" formerly ascribed to the presence in it of vitamin A might, in

reality, be due to whatever constituted the difference between it and the "vitamin-free casein" which we have described in recent papers. We accordingly made comparisons in different ways between "light white casein" extracted as we were in the habit of extracting it and "light white casein" not treated in any way. We also compared untreated "light white casein" with some of the same batch heated in exactly the same way as Messrs Glaxo heat their caseinogen to make the "vitamin-free casein." We are satisfied that the extraction of the "light-white casein" is entirely unnecessary. It follows that the heating process of Messrs Glaxo is therefore unnecessary and moreover we have shown that it is slightly but definitely harmful to the caseinogen.

Method.

1. *Exp. 1. Comparison of caseinogens as the protein of a vitamin A-free diet.*

A large batch (2 cwts.) of "light white casein" (B.D.H.) was thoroughly mixed. A part of it was left untreated, a second part of it was extracted once with alcohol, 4 times with ether, cold (24 hours each extraction) and a third part was, through the courtesy of Messrs Glaxo, heated in thin layers as they heat their own caseinogen to render it "vitamin-free." The three parts are here referred to as

(*a*) untreated "light white casein";
(*b*) extracted "light white casein";
(*c*) treated "light white casein."

The three forms of caseinogen were incorporated in vitamin A-free diets in the usual way (*i.e.* 15 % caseinogen, 73 % dextrinised rice starch, 8 % dried yeast and 4 % (Steenbock's 40) salt mixture; the diet was given to the rats *ad lib.* together with about 2 units vitamin D per rat twice a week). The three diets were tested on large groups of animals to determine (*a*) the average length of time required for the animals to cease growing, (*b*) the average weight at which the animals ceased growing. Table I gives a summary of the results of this experiment. Details are given in Tables II, III and IV. It is evident that (*a*) the times taken for groups of animals to cease growing and (*b*) the average weights at which these animals cease growing are the same

Table I. *Summary of results of Exp. 1.*

To compare the growth of groups of rats given a vitamin A-free diet containing
(*a*) untreated "light white casein" (B.D.H.),
(*b*) extracted "light white casein" (B.D.H.) (once alcohol, 4 times ether),
(*c*) treated "light white casein" (B.D.H.) (heated in thin layers).

		Average of highest weights attained g.	Average weights at which considered steady g.	Average of times taken for weights to become steady (days)
(*a*) Untreated L.W.C.	♂	110·0	102·8	41·9
	♀	105·5	99·7	53·8
(*b*) Extracted L.W.C.	♂	110·7	102·8	38·9
	♀	101·6	96·1	43·8
(*c*) Treated L.W.C.	♂	82·1	77·1	34·2
	♀	81·8	77·1	38·5

whether untreated or extracted "light white casein" is used in the basal diet. On the other hand, the animals whose basal diet contained the treated caseinogen ceased growing in a shorter time and at a lower weight than either of the other two groups. Two interpretations of this are possible. Either the "light white

Table II.

To show: (*a*) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing untreated "light white casein."
(*b*) the average weight at which these rats became steady.
(*c*) the effect of giving 5 of these rats a daily dose of 0·1 g. of a vitamin margarine for 5 weeks.
"Light white casein," untreated in the vitamin A-free diet.

Rat	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Dose	Response in 35 days g.
♂ M 4804	50	133	132	45	—	—
♂ M 4853	43	139	136	59	—	—
♂ M 4852	49	139	128	50	—	—
♂ M 4898	45	132	109	49	—	—
♂ M 4900	47	125	114	42	—	—
♂ M 4856	46	113	112	45	—	—
♂ M 4855	47	137	117	48	—	—
♂ M 4899	41	93	91	45	—	—
♂ L 2	48	170	162	78	—	—
♂ 2323	38	105	98	36	—	—
♂ M 4896	47	109	97	37	—	—
♂ M 4854	42	72	70	24	0·1 g. G. Ch. marg.	43
♂ M 4810	44	82	72	24	0·1 g. G. Ch. marg.	48
♂ 2342	46	54	52	22	0·1 g. G. Ch. marg.	69
♂ M 4809	42	109	105	42	—	—
♂ M 4806	46	85	84	42	—	—
♂ M 4897	46	163	155	63	—	—
♂ M 4851	44	53	46	20	0·1 g. G. Ch. marg.	−3, dead* in 2 days
♂ M 4811	46	77	73	24	0·1 g. G. Ch. marg.	42
♂ M 4895	(56)	(82)	(68*)	(42)	—	—
	Av. = 45·1	Av. = 110·0	Av. = 102·8	Av. = 41·9		
♀ M 4864	49	110	107	56	—	—
♀ M 4803	44	109	90	66	—	—
♀ M 4865	44	124	120	67	—	—
♀ L 5	44	120	115	77	—	—
♀ M 4868	45	101	97	46	—	—
♀ M 4867	(47)	(125)	Discarded	(76)	—	—
♀ M 4804	41	111	103	42	—	—
♀ M 4802	42	105	104	50	—	—
♀ M 4807	42	93	91	42	—	—
♀ M 4808	45	85	80	42	0·1 g. G. Ch. marg.	16
♀ M 4805	40	97	90	50	—	—
♂ M 4866	(44)	—	(38*)	(18)	—	—
♀ L 6	(36)	—	(36*)	(13)	—	—
	Av. = 43·6	Av. = 105·5	Av. = 99·7	Av. = 53·8		Av. + 43·6 (omitting*)

* Rat died, not included in averages.

casein" contains vitamin A which has not been removed by extraction but has been destroyed by heat, or the "light white casein" does not contain any vitamin A and the heat has damaged it as protein or some unknown factor in it. The later experiments throw light on this matter.

2. *Exp. 2. The result of substituting untreated "light white casein" for (a) extracted "light white casein" or (b) treated "light white casein" in the vitamin A-free diets of rats which have ceased to grow on these diets.*

(*a*) Fifteen rats (6 bucks and 9 does) which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given a diet containing untreated "light white casein," to see whether the amount

Table III.

To show: (*a*) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing extracted "light white casein";
(*b*) the average weight at which these rats became steady;
(*c*) the effect of substituting untreated "light white casein" for extracted "light white casein" in this diet.

"Light white casein" in a vitamin A-free diet.

	Extracted				Untreated		
Rat	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)
♂ M 4876	52	94	85	41	1	− 9	13
♂ M 4904	50	129	117	38	1	−16	12
♂ M 4873	51	111	108	40	—	−20	16
♂ M 4875	47	112	103	40	—	−36	13
♂ M 4906	55	126	122	38	—	−10*	18
♂ 2325	38	92	82	36	—	−10*	2
♂ M 4902	(46)	(126)	(104*)	(35)	—	—	—
♂ M 4903	(50)	(101)	(84*)	(24)	—	—	—
♂ M 4874	(43)	(43)	(32*)	(28)	—	—	—
♂ M 4905	(49)	(49)	(39)	(15)	Died within 6 days of being put on a test; therefore discarded		
♂ 2344	(43)	(44)	(40)	(18)	Found wandering in rat room; therefore discarded		
♂ M 4901	(40)	(40)	(34*)	(14)	—	—	—
	Av. = 48·8	Av. = 110·7	Av. = 102·8	Av. = 38·9			
♀ M 4862	48	128	125	43	5	−11	30
♀ M 4870	46	91	89	46	14	13	24
♀ M 4871	51	85	82	40	—	− 8	21
♀ M 4861	52	125	117	38	—	−17	21
♀ M 4863	42	94	82	49	3	−10	14
♀ M 4860	45	90	84	43	—	−18	9
♀ M 4872	49	85	81	46	—	−26*	7
♀ M 4869	50	88	82	40	—	−22*	15
♀ M 4858	45	128	123	49	7	−13	31
♀ M 4857	(47)	(47)	(36*)	(17)	—	—	—
	Av. = 47·5	Av. = 101·6	Av. = 96·1	Av. = 43·8			

* Rat died, not included in averages.

of vitamin which might be present in the untreated sample would be sufficient to make the rats resume growth. The result has been summarised in Table III. It may be seen that 4 rats have increased in weight (5, 14, 3, 7 g. respectively), 2 have gained 1 g. which is not considered significant, 9 have lost weight. It is evident that the amount of vitamin A, if any, in untreated "light white casein" is negligible.

(*b*) Nineteen rats (7 bucks and 12 does) which had become steady in

weight on a vitamin A-free diet containing treated "light white casein" were given a diet containing untreated "light white casein." The response to this change was greater than the response when extracted was changed to untreated "light white casein." Seven rats increased in weight (11, 9, 14, 19, 16, 2, 5 g. respectively) and the rest lost weight or gained only 1 g.

Table IV.

To show (*a*) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing treated "light white casein";
(*b*) the average weight at which these rats became steady;
(*c*) the effect of substituting untreated for treated "light white casein" in the diet;
(*d*) the effect of giving 5 of these rats a daily dose of 0·1 g. of a vitamin margarine for 5 weeks.

"Light white casein" in vitamin A-free diet.

	Treated				Untreated				
Rat	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)	Dose	Response in 35 days g.
♂ M 4879	44	85	82	34	11	+10	16	—	—
♂ M 4800	44	100	95	43	—	−17	16	—	—
♂ M 4798	47	92	79	34	1	− 7	25	—	—
♂ M 4878	51	60	57	32	—	− 5	17	—	—
♂ L 3	39	80	75	50	9	+ 5	16	—	—
♂ 2324	38	64	64	36	—	−12*	8	—	—
♂ M 4797	49	100	97	42	—	−30*	18	—	—
♂ L 1	49	61	56	24	—	—	—	0·1 g. G. Ch. marg.	27
♂ M 4801	43	90	80	28	—	—	—	0·1 g. G. Ch. marg.	9
♂ M 4799	49	100	96	29	—	—	—	0·1 g. G. Ch. marg.	24
♂ M 4796	48	71	67	24	—	—	—	0·1 g. G. Ch. marg.	35
♂ M 4880	(53)	(112)	(102*)	(32)	—	—	—	—	—
♂ M 4877	(50)	(111)	(105*)	(32)	—	—	—	—	—
	Av. = 45·5	Av. = 82·1	Av. = 77·1	Av. = 34·2					
♀ M 4888	44	74	69	31	14	+ 1	21	—	—
♀ M 4850	51	111	108	36	—	− 5	16	—	—
♀ M 4846	44	94	88	43	—	− 9	27	—	—
♀ M 4891	50	93	86	43	19	− 1	23	—	—
♀ M 4849	52	112	110	45	16	+ 5	28	—	—
♀ M 4848	49	85	82	43	—	−10	13	—	—
♀ L 4	37	64	62	45	—	−12*	11	—	—
♀ M 4890	42	59	50	24	—	− 5*	3	—	—
♀ 2343	44	68	67	39	2	−12*	12	—	—
♀ M 4887	45	77	74	31	—	− 2*	14	—	—
♀ M 4845	47	76	76	38	5	−11*	11	—	—
♀ M 4844	48	48	45	17	—	—	—	0·1 g. G. Ch. marg.	−1
♀ M 4892	47	102	95	66	—	−10	3	—	—
♀ M 4893	(43)	(78)	(56*)	(24)	—	—	—	—	—
♀ M 4847	(49)	(102)	(86*)	(49)	—	—	—	—	—
♀ M 4889	(53)	(68)	(53*)	(24)	—	—	—	—	—
	Av. = 40·2	Av. = 81·8	Av. = 77·1	Av. = 38·5					

* Rat died, not included in averages.

It is concluded that there is less difference between the extracted and untreated caseinogen than between the treated and untreated. Indeed the difference between the extracted and untreated is considered insignificant.

3. *Exp. 3. The effect of giving equal doses (0·1 g.) of a vitamin margarine to rats which had ceased to grow on a vitamin A-free diet containing (a) untreated "light white casein" and (b) treated "light white casein."*

Four bucks and one doe were used in each part of this experiment. (A fifth buck in one group died after 3 days and was therefore discarded.) The result is summarised in Table V. Details are given in Tables II and IV.

This result is open to the interpretation that the small amount of vitamin A which might be present in the untreated "light white casein," although not enough to prevent cessation of growth, might supplement the vitamin A of the vitamin margarine and thus cause the greater growth of this group of animals. This explanation, however, seems to be impossible in view of the work described in earlier papers, for we have found that even when excessive amounts of vitamin A in the form of 0·2 g. cod-liver oil daily have been given, we have obtained remarkable increases in growth rates on substituting "light white casein" (B.D.H.) for the "vitamin-free casein" (Glaxo). It seems much more probable that the treated caseinogen has been altered in some way so that it is less adequate for growth than the untreated form.

Table V.

Caseinogen	Dose of vitamin margarine g.	Average increase in weight of the group in 5 weeks g.
Untreated	0·1	43·6
Treated	0·1	18·8

4. *Exp. 4. The effect of giving cod-liver oil to rats which have become steady in weight on a vitamin A-free diet containing* (*a*) *untreated "light white casein" and* (*b*) *extracted "light white casein."*

Where the same dose of cod-liver oil has been used for comparing the two forms of caseinogen, results are, of course, directly comparable. Where different doses have been used, the results are compared by reference to a curve relating response to dose of oil. The curve which we have worked out for our own laboratory has already been described [Coward, Key, Dyer and Morgan, 1930]. The equation for this curve is $y = 12{\cdot}1 + 40{\cdot}24 \log x$, where y = the mean increase of a group of animals all given the same dose, and x = the dose of cod-liver oil in mg. It has been found that this curve can be resolved into two somewhat similar curves, one for the bucks used in its construction and one for the does. These curves are:

$$y = 11{\cdot}3 + 50{\cdot}3 \log x \text{ (bucks).}$$

and

$$y = 12{\cdot}4 + 27{\cdot}4 \log x \text{ (does).}$$

When it has not been possible to use equal numbers of bucks and does for a test, comparisons may be made by means of the two curves. The average of the results will then be more accurate than a direct comparison made through the original curve. We now express the vitamin A potency of any sample examined in our laboratory as a percentage of the potency of the oil which we used in the making of these curves and which we have temporarily adopted as a standard. We find the mean increase of a group of rats (about 10) given a certain dose of cod-liver oil daily for 5 weeks, then find by the curves what dose of our standard oil would have given that increase and thus determine the relative potency of the oils. In this way we found two oils, B and C, to have the same vitamin A potency relative to oil A whether they were tested on a diet containing untreated "light white casein" or on one containing extracted "light

white casein." Table VI gives a summary of these figures and Table VII gives the details.

This result is regarded as additional evidence that the amount of vitamin A in "light white casein," if any, is negligible, and that the laborious and expensive process of extracting "light white casein" is superfluous.

Table VI.

Oil	Potency expressed as a percentage of the potency of oil A when examined on basal diets containing (*a*) Untreated caseinogen	(*b*) Extracted caseinogen
B	83·05	72·9
C	173·8	189·6

Table VII.

To show that the vitamin A potency of an oil relative to another oil is the same whether determined with untreated "light white casein" or with extracted "light white casein" in the basal diet.

Oil	Dose mg.	No. of bucks	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	No. of does	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	Relation to oil A (%) according to Bucks	Does
B	1·0 untreated caseinogen	10	4·2	0·[illegible]	9	11·8	0·95	72·3	95
							Average	=83·65	
							Weighted mean	=83·05	
	4·0 extracted caseinogen	4	35·75	3·00	5	24·6	2·79	76·5	69·7
							Average	=73·1	
							Weighted mean	=72·9	
C	1·0 untreated caseinogen	6	27·7	2·118	13	20·2	1·9275	211·8	192·75
	5·0 untreated caseinogen	7	54·9	7·357	11	36·2	7·393	147·1	147·9
							Average	=174·9	
							Weighted mean	=173·8	
	5·0 extracted caseinogen	13	61·4	9·91	4	37·25	8·075	198·2	161·5
							Average	=179·85	
							Weighted mean	=189·6	

SUMMARY.

It has been shown in several ways that "light white casein" (B.D.H.) may be safely used without extraction for vitamin A tests.

1. Large groups of rats were given a vitamin A-free diet containing (*a*) extracted or (*b*) non-extracted "light white casein" (B.D.H.). They became steady in weight at about the same weight and in about the same length of time.

2. Rats which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given an exactly similar diet

but containing non-extracted "light white casein"; they did not resume growth but continued to lose weight.

3. The estimation of the vitamin A potency of a sample of cod-liver oil relative to another sample used as a standard of reference in these laboratories was the same whether extracted or non-extracted "light white casein" was used in the basal diet.

The extraction of the caseinogen has not removed the growth-factor whose existence has recently been demonstrated by Coward *et al.* [1929].

The heating of "light white casein" by Messrs Glaxo in the way in which they prepare their "vitamin-free casein" only partially destroyed this factor.

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APPENDIX.

The method of determining the equations of the curves.

The mean increases (y) of the groups of rats given different doses of cod-liver oil for a period of 5 weeks were plotted against the logarithms of the doses of oil given. They fell practically on a straight line. The best straight

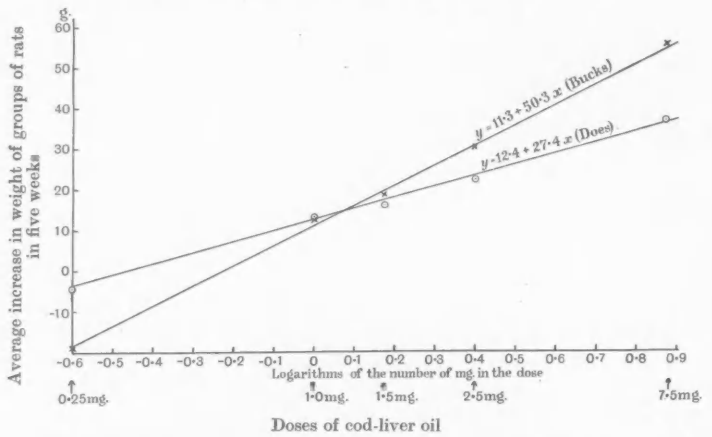

Fig. 1. Curves relating mean increases in weight in 5 weeks of groups of rats (bucks and does respectively) to the logarithms of the doses of cod-liver oil given.

The more nearly the mean increases in each curve lie in a straight line when plotted against the logarithm of the dose, the more truly is the response expressed by the corresponding logarithmic equation.

line through the five points was obtained by the method described by Fisher [1925, p. 117].

$$y = a + b(x - \bar{x})$$

where $a = \bar{y} =$ mean value of y

$\bar{x} =$ mean value of x

and $$b = \frac{\Sigma y(x - \bar{x})}{\Sigma (x - \bar{x})^2}.$$

This gave the equation $y = 12{\cdot}1 + 40{\cdot}24x$ (where x is the logarithm of the dose of oil), whence is derived the equation $y = 12{\cdot}1 + 40{\cdot}24 \log x$ to represent the relation between response and dose of oil.

This equation was obtained from results on groups of rats consisting of approximately equal numbers of bucks and does. It occurred to us that it might be useful to have separate equations for results from bucks and does respectively. We therefore plotted the mean increases of the bucks and does in each group separately against the logs of the doses. These also gave two practically straight lines (Fig. 1) which were determined by the same method:

$$y = 11{\cdot}3 + 50{\cdot}3\,x \text{ (bucks)},$$

and $$y = 12{\cdot}4 + 27{\cdot}4x \text{ (does)}.$$

The equations relating response to dose are therefore:

$$y = 11{\cdot}3 + 50{\cdot}3 \log x \text{ (bucks)},$$

and $$y = 12{\cdot}4 + 27{\cdot}4 \log x \text{ (does)}.$$

Two points of interest arise from a consideration of these curves. Firstly, only about 15 animals were used for each point on the curve. The smoothness of the curve would appear to indicate therefore that a very reliable average can be obtained from 15 animals, a comparatively small number for a biological estimation. Secondly, the two curves indicate that the bucks respond more vigorously than the does to the higher doses of vitamin A but it is remarkable that the does respond more vigorously than the bucks to the lower doses. This led us to wonder whether the mortality among the bucks had been greater than that among the does in work on vitamin A deficiency. We therefore counted the numbers of bucks and does respectively that had died during the test period (5 weeks) of the experiment and also those from similar experiments that we have just concluded. We found that, of 288 bucks which had been used for testing various doses of cod-liver oil, 57 or 19·8 % had died, while of 277 does used in the same experiments (in more or less equal numbers on each test) 37 or 13·4 % had died. This difference appears to be significant.

Dr J. Gaddum of the National Institute for Medical Research has made the following calculation from the data and has concluded that the difference is significant and, therefore, that male rats require more vitamin A to prevent death than do female rats.

Group of rats	Dose of cod-liver oil given daily mg.	No. of bucks in group	Total no. of bucks dying during test	% no. of bucks dying during test	No. of does in group	Total no. of does dying during test	% no. of does dying during test
1	1 oil B	12	2	16·7	10	1	10·0
2	4 „ B	5	1	20·0	5	0	0·0
3	0·5 „ C	11	4	36·4	14	2	14·3
4	1·0 „ C	9	3	33·3	14	1	7·1
5	5·0 „ C	14	1	7·1	4	0	0·0
6	5·0 „ C	7	0	0·0	12	1	8·3
7	0·5 „ D	9	1	11·1	13	1	7·7
8	1·0 „ D	10	2	20·0	13	2	15·4
9	0·5 „ E	13	3	23·1	16	0	0·0
10	1·0 „ E	13	3	23·1	13	2	15·4
11	0·1 „ F	17	7	41·2	11	0	0·0
12	0·5 „ F	9	1	11·1	13	1	7·7
13	1·0 „ F	16	2	12·5	6	0	0·0
14	1·0 „ G	11	3	27·3	11	3	27·3
15	1·0 „ H	10	0	0·0	12	3	25·0
16	1·0 „ J	11	1	9·1	12	0	0·0
17	1·0 „ K	2	0	0·0	9	1	11·1
18	0·25 „ A	18	11	61·1	13	6	46·2
19	1·0 „ A	23	6	26·1	14	7	50·0
20	1·5 „ A	13	3	23·1	22	5	22·7
21	2·5 „ A	21	2	9·5	11	0	0·0
22	7·5 „ A	19	1	5·3	12	0	0·0
23	20·0 „ A	15	0	0·0	17	1	5·9
		288	57		277	37	

If the sexes were equally sensitive, the number of groups in which the mortality of the males was larger than that of the females should equal the number of groups in which the mortality of the females was larger than that of the males. Actually, more males than females died in 17 groups, more females than males died in 5 other groups and in 1 group the numbers of males and females that died were equal. Thus:

	More males dead	More females dead
Observed	17·5 (a)	5·5 (b)
Calculated on assumption that mortalities are equal	A (c)	A (d)

where A is a very large number.

To find the probability that these two results are compatible [see Fisher, 1925, p. 84]:

$$\chi^2 = \frac{(ad-bc)^2\,(a+b+c+d)}{(a+b)\,(c+d)\,(a+c)\,(b+d)}$$

$$= \frac{[A\,(17{\cdot}5-5{\cdot}5)]^2\,(2A)}{23\times 2A\times A\times A} \quad \text{(neglecting } a+b \text{ in comparison with the large number } A\text{)}$$

$$= \frac{12^2}{23} = 6{\cdot}26.$$

The table of χ^2 shows (when $n = 1$) that the probability of this value of χ^2 being due to chance is between 1/50 and 1/100.

LXV. THE RELATION BETWEEN AMYLASE AND LACTIC ACID FORMATION IN MUSCLE.

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The work reported in this paper was begun with the intention of investigating further the nature of the inhibition of lactic acid formation in muscle which is brought about by pancreatic and other extracts. This phenomenon had been shown by Case and McCullagh [1928] to be caused by the amylase present in such extracts, and it was demonstrated that similar inhibition could be brought about by amylase from various other sources, both animal and plant. In an earlier paper the mode of action of the "pancreatic factor," then unidentified with amylase, had been described by McCullagh [1928] as a suppression of the esterification of phosphoric acid; in other words, as a prevention of the formation of hexosephosphates rather than a prevention of their breakdown to lactic acid. This hypothesis was adhered to in the subsequent communication.

Harrison and Mellanby [1930] agreed that the substance responsible for the inhibition is amylase, but they did not regard as admissible the above interpretation of the manner in which it acts. Actually it appears that their criticism was based, in large part, upon a misunderstanding with regard to the meaning which was intended to be borne by the explanation in question.

It is clear that a statement such as that "inhibition of lactic acid formation depends upon the suppression of esterification of carbohydrate and phosphoric acid" can be presumed to apply only to those cases where inhibition does in fact occur; that is to say, to cases where the carbohydrate involved is starch or glycogen. When activated glucose is the substrate, amylase causes no inhibition, as was shown both by Case and McCullagh and by Harrison and Mellanby. From these circumstances, taking into consideration the fact that starch and glycogen are natural substrates for amylase, while glucose and other hexoses are not, it seems a reasonable deduction that the inhibition observed is related to an interaction of some kind between polysaccharide and amylase, in virtue of which the lactic acid-forming process is checked at its first stage, namely that of esterification. In a sense, it may be said that Harrison and Mellanby are agreeing to this when they put forward the suggestion that "an explanation of the experimental observations can be based

on the well-known fact that amylase breaks down starch to maltose," which substance as Meyerhof has shown is practically unacted upon by the muscle enzyme system. This explanation, which as Harrison and Mellanby point out seems obvious enough at first sight, is in the present writer's opinion inadequate, in such a simple form, to account for the complete and instantaneous inhibition of lactic fermentation which is produced by very small amounts of added amylase. It is not conceivable that immediately after the addition to the system of amylase all the starch is at once transformed to maltose and in that way removed from the sphere of action of the esterifying and other enzymes. It seems much more likely that the explanation is to be sought in the formation of some amylase-starch compound or complex, whereby the starch is protected from and rendered inaccessible to the muscle enzymes. That something of this nature indeed takes place is suggested by the fact that even in the presence of considerably more amylase than is necessary to produce complete inhibition of esterification and lactic acid formation, the characteristic blue compound with iodine can be obtained, thus demonstrating the continued existence in the solution of unhydrolysed starch. The breakdown of the starch into maltose proceeds slowly and can be followed by the iodine method or a reduction method, while the lactic acid formation is completely checked *ab initio*.

The experimental work to be described was originally commenced in order to produce fresh justification for this point of view, but as will be seen, results were obtained which throw light from a different angle on the problem of the relation between amylase and lactic acid formation in muscle.

Experimental.

In order to test the theory of Harrison and Mellanby referred to above, namely that maltose formation is responsible for the inhibition, the following experiment was designed. If one were to incubate starch for a time with amylase in sufficient quantity to cause complete inhibition of lactic acid formation by a given muscle extract, and then, after this preliminary incubation, remove or destroy the amylase, it should be possible to show that there still remains starch which can now yield lactic acid when incubated with muscle extract.

Table I sets forth the results of one experiment carried out on these lines.

Table I.

			mg. lactic acid in 15 cc. sample		
			Before incubation	After incubation	Change
1.	Muscle extract + starch + buffer + water		10·9	27·8	16·9
2.	" " + 5 cc. amylase		11·1	12·3	1·2
3.	" (starch + buffer + 5 cc. amylase)	A	11·2	28·8	17·6
4.	" " "	B	10·9	23·3	12·4
5.	" " "	C	10·9	18·2	7·3
6.	" " "	D	11·2	14·6	3·4
7.	" " "	E	11·3	13·9	2·6

In preparation A the starch, buffer and amylase were mixed and incubated together at 27° for 1 minute, then boiled for 5 minutes in order to destroy the amylase. B, C, D and E received similar treatment, the incubation times being 5, 10, 20 and 30 minutes respectively before the 5 minutes' boiling. All were cooled to room temperature before the muscle extract was added. The composition of the final mixture was in all cases:

25 cc. muscle extract,
10 cc. 2 % starch,
10 cc. phosphate buffer,
5 cc. water or amylase.

Incubation was carried out for half an hour at 27°. The technique of sampling and of estimation of lactic acid was exactly as described in former work [Case, 1929], as was the preparation of the muscle extract and the composition of the buffer. This applies to all the lactic acid experiments reported in this paper.

The amylase employed was a diluted preparation of saliva, 5 cc. of which had been shown by a preliminary test to give 100 % inhibition of lactic acid formation with an average muscle extract.

It follows from these results that for a period of at least half an hour after the addition of amylase, starch remains present in the system and can be utilised by the lactic acid-forming enzymes if the amylase is removed. It would seem then that the starch is in some way held by the amylase prior to any destruction, and held in such a way that it is inaccessible to the myozymase system. It was a conception of this kind that was foreshadowed in the concluding sentence of Case and McCullagh's [1928] paper, *viz.* "it may be that some molecular union between amylase and its substrate, prior to enzymic hydrolysis, can be demonstrated."

All that has been said above applies with equal force to the case of glycogen, which of course is the naturally-occurring substrate for the muscle enzymes. An experiment similar in every respect to that of which Table I is the protocol, except that starch was replaced by glycogen, yielded substantially identical results.

It is well known that the tissue of numerous organs, including muscle, contains amylase in varying quantities. With regard to muscle, it becomes of interest to inquire as to the nature of the rôle played by its amylase.

Ronzoni [1929] believes that "hydrolysis of starch is a necessary step in glycolysis of muscle extract." In support of this view, she claims to have produced an acceleration of esterification by adding amylase to muscle extracts which, owing to short periods of extraction, were deficient in this enzyme. The present writer has, however, never succeeded in obtaining confirmation of this. It is interesting to note in this connection that preparations of amylase from muscle itself, made by glycerol extraction or other means, resemble amylases from other sources in that their addition in sufficient quantity to

the myozymase system produces inhibition of lactic acid formation. This fact was realised by Ronzoni and is mentioned in her paper.

Now the muscle enzyme system as a whole is strikingly thermolabile. Heating for a few minutes at 37° is sufficient to destroy its power of forming lactic acid from carbohydrate, or of esterifying phosphate. Muscle-amylase itself has a much greater degree of stability. It should be possible therefore, by heating muscle extract for, say, a quarter of an hour at 37°, to leave the amylase activity intact while destroying the lactic acid-forming property of the extract. A comparison of the rates of starch or glycogen destruction in an extract so treated, and at the same time in an unheated portion of the same extract, should then give significant results. If in both cases carbohydrate disappeared at the same rate, the fact would be an indication that amylase action is the first step in the degradation of starch. In order to render such a conclusion admissible, it would be necessary to ensure that the starch concentration was small enough to be below the saturation value for the amount of enzyme present.

In Table II are embodied the results of a number of experiments made to test this point. In each instance a part of the muscle extract was heated for 15 minutes at 37°, the remainder being kept at 0°. After cooling the first portion to 0° again, incubation mixtures were set up consisting of 20 cc. of muscle extract, 10 cc. of phosphate buffer and 10 cc. of 0·4 % starch. The final concentration of starch was thus 0·1 %. The incubations were carried out at 27°, and at intervals 0·5 cc. samples were tested with iodine. In each case the time was noted at which the addition of iodine produced no coloration.

Table II.

		Time (in mins.) for achromic point with iodine				Time (in mins.) for achromic point with iodine	
	p_H	Normal extract	Heated extract		p_H	Normal extract	Heated extract
A	6·0	41	44	C	6·0	35	35
	7·0	37	38		7·0	36	39
	8·0	32	32		8·0	30	33
B	6·0	20	21	D	6·0	13	12
	7·0	20	18		7·0	13	14
	8·0	18	20		8·0	12	13

A, B, C and D represent different muscle extracts made at different times.

Variations of p_H between the above limits, although in each case altering to some extent the actual times taken for the attainment of the achromic point, are seen to make no difference to the relative values for heated and unheated extracts.

The important point emerging from these figures is that in every instance the rates of disappearance of starch are the same, in spite of the fact that in one case lactic acid is being produced in the ordinary way, while in the other case nothing is occurring beyond amylolytic hydrolysis of the starch. This

seems to furnish strong evidence in support of the conclusion that the breakdown of starch to lactic acid depends primarily upon the amylase present in the extract.

Table III demonstrates that lactic acid production is, in fact, eliminated in the heated extracts. The figures given are for incubation mixtures A to D of the previous table, the p_H being 7·0.

Table III.

	Lactic acid production in 1 hour (mg. in 15 cc. sample)	
	Normal extract	Heated extract
A	4·9	0·5
B	7·0	0·8
C	5·5	0·5
D	8·7	1·0

The figures here given for "heated extract" are not quantitatively reliable, on account of the fact that the amounts of lactic acid are too small to be estimated accurately. The significance of the table, however, is not thereby affected.

A somewhat similar series of experiments was next performed, with the difference that instead of starch, glycogen was employed, in a concentration of 0·1 %. As before, normal and heated extracts were incubated with buffer (p_H 7·0) and glycogen. Instead of following glycogen disappearance by an iodine method, 5 cc. samples were taken initially and at periods of 5, 10 and 30 minutes. The glycogen in these was estimated by the modification of Pflüger's [1909] method described by Barbour [1929]. The samples were delivered directly into 5 cc. of hot potassium hydroxide solution, thus preventing any further enzymic activity.

The results of these experiments are given in Table IV. As in Table II, E, F and G are separate muscle extracts.

Table IV.

	% glycogen in solutions							
	Normal extract				Heated extract			
Period of incubation in minutes ...	0	5	10	30	0	5	10	30
E	0·094	0·077	0·061	0·005	0·095	0·079	0·060	0·004
F	0·096	0·081	0·069	0·024	0·096	0·080	0·070	0·030
G	0·096	0·070	0·048	0·000	0·097	0·068	0·050	0·000

Here again we find a parallel disappearance of glycogen in the corresponding cases where the muscle enzyme has and has not been heated for 15 minutes at 37°; these results therefore corroborate those of Table II.

If the conclusion just drawn is correct, it is to be expected that any agency which suppresses the amylolytic activity of a muscle extract would suppress in corresponding degree the formation of lactic acid. Actually to remove the

amylase without affecting the other components of the system would be a difficult task; fortunately, however, another way out of the difficulty is open.

It has been known for some time that muscle-amylase, in common with liver-amylase, differs in some respects from the enzyme obtained from pancreas, saliva, malt and other sources. Barbour [1929] made a study of glycogenolysis by muscle-amylase, and found that the sole product is a trisaccharide, which seems not to be identical with any sugar hitherto described; though Lohmann [1926] had reported among the products of glycogenolysis in muscle extracts a trisaccharide which may have been the same as that described by Barbour. Lohmann at the time suggested that the substance might be identical with the amylotriose of Pringsheim, but Barbour's more complete investigation of its properties appears to render this unlikely. If glycogen is hydrolysed with pancreatic or salivary amylase the products consist mainly of *iso*maltose with a little glucose; no trisaccharide is formed.

It was found possible to confirm Barbour's work, and by starting with several grams of glycogen to obtain relatively large amounts of a substance answering to his description of the trisaccharide. In general, glycerol extracts of finely minced rabbit muscle were employed and were incubated for 24 hours or longer with phosphate buffer (p_H 6·5) and 5 % glycogen. The trisaccharide was isolated from the digest according to the directions given by Barbour.

Now Wohl and Glimm [1910] showed that in the case of malt-amylase, the addition of maltose, the chief product of hydrolysis, caused inhibition of the amylolytic activity when starch was the substrate; this fact was made use of by Case and McCullagh [1928]. In a similar way, addition to muscle-amylase of the trisaccharide referred to above should depress the activity of the amylase. That this is so was shown by Barbour, and the following figures obtained during the course of the present work afford confirmation.

Table V.

Incubation period (hours)	Glycogen content (%)			Hydrolysis (%)	
	0	5	20	5	20
Control	0·98	0·64	0·15	35	85
Trisaccharide	0·97	0·95	0·97	2	0
Glucose	0·97	0·66	0·20	32	79
Maltose	0·98	0·66	0·23	33	77
Sucrose	0·96	0·68	0·22	29	77

In this experiment each flask contained 5 cc. of muscle-amylase (glycerol preparation) and 5 cc. of 2 % glycogen (made up in phosphate buffer of p_H 7·0). The different sugars (0·5 g. of each) were dissolved in the glycogen solutions before mixing with the enzyme. The mixtures were incubated at 27° and the glycogen was estimated in 3 cc. samples by the method referred to earlier in the work.

It is clear that the trisaccharide exerts a marked specific inhibitory action upon muscle-glycogenase.

The next step was to try the effect of adding trisaccharide to incubation

mixtures of glycogen and myozymase. To this end tubes were set up as follows (S represents trisaccharide):

1. 10 cc. muscle extract + 5 cc. 2 % glycogen + 5 cc. buffer
2. ,, ,, ,, + 0·25 g. S
3. ,, ,, ,, + 0·5 g. S
4. ,, ,, ,, + 1·0 g. S
5. ,, ,, ,, + 1·0 g. glucose
6. ,, ,, ,, + 1·0 g. maltose
7. ,, ,, ,, + 1·0 g. sucrose
8. ,, + 5 cc. water ,, + 0·25 g. S
9. ,, ,, ,, + 0·5 g. S

The production of lactic acid during one hour at 27° is shown in Table VI.

Table VI.

Tube No.	mg. lactic acid in 10 cc. sample		
	Before incubation	After incubation	Change
1	6·5	19·4	12·9
2	6·4	16·8	10·4
3	6·7	7·4	0·7
4	6·5	7·1	0·6
5	6·5	17·2	10·7
6	6·2	16·9	10·7
7	6·4	18·5	12·1
8	6·8	6·9	0·1
9	6·4	6·9	0·5

Here again it is seen that the addition of trisaccharide in a concentration of 2·5 % has completely suppressed the formation of lactic acid from glycogen, while the other sugars tried have had no such effect. Tubes 8 and 9 are intended to demonstrate that the trisaccharide itself is not acted upon by muscle enzymes.

This experiment was repeated using half quantities throughout, and with the addition of sodium fluoride in 0·02 *M* concentration. Instead of lactic acid, phosphates were determined before and after the incubation by the Briggs-Bell-Doisy colorimetric method. The results, as is apparent from the next table, bear out those of Table VI; that is to say, esterification has been inhibited in the systems containing the trisaccharide, but in no others.

Table VII.

Tube No.	mg. P as free phosphate in 3 cc. sample		
	Before incubation	After incubation	Change
1	1·02	0·40	−0·62
2	1·04	0·52	−0·52
3	1·03	1·00	−0·03
4	1·03	1·02	−0·01
5	1·04	0·55	−0·49
6	1·02	0·57	−0·45
7	1·01	0·54	−0·47
8	1·05	1·02	−0·03
9	1·05	1·03	−0·02

Discussion.

Considering jointly the significance of the experimental results embodied in Tables V, VI and VII, one is led to the conclusion that since lactic acid production in muscle is inhibited by the trisaccharide, and the trisaccharide inhibits the activity of muscle-glycogenase, of whose action upon glycogen it is the end-product, then the first stage in the breakdown of glycogen into lactic acid is one dependent upon amylolytic activity. This is the same conclusion that was arrived at from a consideration of the facts reported in Tables II, III and IV, and up to a point is in agreement with Ronzoni's view of the situation. But whereas Ronzoni postulates amylolytic hydrolysis as a necessary preliminary, the present writer is not of this opinion. As has been said, it seems fairly certain from Barbour's work that the only product of glycogenolysis by muscle-amylase is the trisaccharide; it appears that the intermediate production of dextrins does not occur. The trisaccharide itself is incapable of being further acted upon by the muscle enzymes, as is shown in Tables VI and VII. It may be said in parenthesis that Lohmann's trisaccharide was also non-fermentable.

The position may then be summarised by the statement that on the one hand the action of muscle-amylase is a necessary preliminary to the formation of lactic acid from glycogen, while on the other hand the product of the hydrolysis of glycogen by muscle-amylase is one which cannot be attacked by myozymase. The inference is therefore that this product, the trisaccharide, is not formed in the process of lactic acid production. Since it is the only product of glycogenolysis by muscle-amylase, it follows that in all probability hydrolysis does not occur at all. The muscle-amylase, if this is the case, is not functioning in the manner that normally would be expected of an amylase.

The suggestion put forward is that we have in muscle a system of enzymes working not only in a coordinated consecutive manner but in a conjoint and simultaneous way, in the sense that the amylase and another component of the system, presumably the esterifying enzyme, are so closely correlated and interdependent in their activity that they may, in fact, be considered to form a single colloid complex or aggregate. It is a generally accepted hypothesis that an enzyme molecule proper is attached to an inactive associated structure, probably of protein. It is necessary to imagine then that in this particular case both the amylase and the esterifying enzyme are attached to the same colloid structure and constitute to that extent a single entity. The first step in the transformation of glycogen to lactic acid seems to be then not merely a union of the carbohydrate with amylase, but a simultaneous union with both amylase and another enzyme component of myozymase—presumably involving two different sites on the carbohydrate molecule. In this connection it is tempting to allude to the extreme thermolability of the myozymase system as a whole. The very pronounced effect of even short exposure to quite low degrees of heat (*e.g.* 37°) is highly unusual, even in enzyme chemistry,

and it is difficult to regard this effect as consistent with the destruction of some enzyme component; it seems a more likely hypothesis that the exaggerated deleterious effect of temperature occurs as the result of the rupture of some delicate colloidal complex between one part of the system and another.

SUMMARY.

1. The nature of the inhibition of lactic acid formation in muscle caused by amylase is further investigated and the conclusion is drawn that an enzyme-substrate compound is formed prior to the hydrolysis of starch or glycogen by amylase.

2. Evidence is adduced in support of the hypothesis that the amylase of muscle is concerned in the process whereby lactic acid is produced from carbohydrate.

3. The concept of two enzymes functioning in a mutually dependent manner and not as individuals is introduced and is briefly discussed with reference to the case of muscle.

I am indebted to Sir F. G. Hopkins for his interest and encouragement, and to Prof. J. B. S. Haldane for much stimulating advice.

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LXVI. THE EFFECT OF STARVATION, PHLORIDZIN, THYROID, ADRENALINE, INSULIN AND PITUITRIN ON THE DISTRIBUTION OF GLYCOGEN IN THE RAT.

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The effect of starvation, phloridzin and diabetes on the main glycogen stores in the liver and skeletal muscles was early established and latterly the effects of insulin and adrenaline on these organs have been intensively studied. A few investigations have been concerned with other organs, such as the heart in diabetes, but both early and recent work has been confined mainly to the liver and skeletal muscles or to the total glycogen content of the body. Thus, although glycogen has been demonstrated in every tissue, we can find no record of an attempt to follow its simultaneous changes in a number of representative organs, and we have made this the subject of the present investigation. Besides the liver and skeletal muscles, therefore, we have studied the heart, the stomach (as representing involuntary muscle), the spleen and the kidney, and a few observations have also been made on the brain, uterus and testicle. The uterus, containing as it does less non-muscular tissue than the stomach, might have been a better representative of involuntary muscle, but for routine purposes it is too small and its use would have limited us to female rats. The kidney and spleen were selected as non-muscular organs with no specialised "glycogen function" such as the liver possesses.

Our first intention was to investigate the glycogen changes in these organs produced by insulin and adrenaline, but a survey of the literature showed that such a comprehensive study had never been undertaken in starvation, or under the action of phloridzin or thyroid and we turned therefore to these first of all.

Technique.

(1) *Animals.* Standard young albino rats (80–150 g.) obtained from the Glaxo laboratories were used throughout. The majority were males. They were fed on an excess of white bread soaked in milk and they increased in weight satisfactorily on this diet. The "fully fed" rats of our experiments have been killed 4 hours after receiving their food in the morning. At this time their

stomachs always contained much partially digested bread. The "24 hours' starved" and "48 hours' starved" animals were similarly fed in the morning, placed in a separate cage with water alone at room temperature 4 hours later, and killed after a further 24 or 48 hours.

(2) *Methods.* The rats were stunned by a blow on the head and immediately decapitated, and the sugar was determined in the blood from the carotid artery. Samples of the representative organs were rapidly removed and plunged into tared tubes containing 1 cc. of boiling 60 % potassium hydroxide. If the stomach contained food it was rapidly washed and dried. The time between death and the immersion of the last sample varied between 40 and 60 seconds, except in the case of the uterus. It took nearly 2 minutes to dissect out this organ cleanly.

We are aware now that this technique shows the glycogen in the muscle to be lower than it really was during life owing to the convulsive struggles of the stunned animal. Our results are however comparable with each other and show clearly the gross changes brought about by starvation *etc.* We do not lay great stress on small differences in the skeletal muscles until they have been confirmed by one or other of the improved techniques now available.

After 3–5 minutes in the boiling potassium hydroxide the tissues were completely dissolved. The tubes were then cooled and weighed, the weight of the sample being obtained by difference. The weights of liver and kidney taken were approx. 1 g.; of the stomach, heart, and skeletal muscles (adductor group of right thigh) 0·5 g. After weighing the tubes were again put into a water-bath at 100° for 3 hours when they were removed, and alcohol was added at once till the final concentration was 80 % (see below). After standing overnight the tubes were centrifuged, and the precipitate was washed twice with 5 cc. of 80 % alcohol *plus* 1 drop of saturated sodium chloride. Dilute hydrochloric acid was then added, and the glycogen was hydrolysed for 3 hours in the usual manner; the liquid was neutralised, made up to a definite volume and the glucose estimated by MacLean's method. It will be seen that the usual micro-Pflüger technique has been followed except in the use of 80 % instead of 66 % alcohol to precipitate the glycogen. This has been shown by Holmes [1929] to give a much more complete precipitation, and when working with very small amounts of glycogen this is a matter of great importance. Table I shows the results of precipitating and washing portions of the same liver (*a*) with 66 % alcohol; (*b*) with 80 % alcohol.

Table I. *Effect of concentration of alcohol on precipitation of glycogen.*

	Glycogen % (liver) of rats starved 24 hours											
												Average
80 % alcohol	0·25	0·26	0·20	0·21	0·20	0·20	0·24	0·30	0·26	0·30	0·25	0·24
66 % „	0·17	0·16	0·12	0·21	0·11	0·09	0·16	0·12	0·12	0·14	0·13	0·14
	Animals fully fed											
80 % alcohol	3·17	2·19	3·08	4·95	4·35	3·56						3·55
66 % „	2·86	2·68	2·79	5·34	3·65	3·52						3·47

In livers with a low glycogen content, the weaker alcohol gives much lower figures, but this difference is not seen when considerable amounts of glycogen are present. The use of the stronger alcohol, therefore, seems important only to remove the last traces of glycogen.

Experiments and results.

(1) *The Control Rats.* (*a*) fully fed; (*b*) 24 hours' starved; (*c*) 48 hours' starved; Table II A.

Although the "standard" rat has often been used for glycogen investigations it has been necessary to establish our own controls especially in the case of the heart, stomach and kidney. The results are given in Table II A. These controls do not apply to the experiments with adrenaline, pituitrin and insulin which were done on larger rats and at another time of year. Further controls for these experiments were performed and the results are tabulated beside the corresponding experiments.

Table II.

A. *Controls.*

Mean % of glycogen and average deviations from the mean.

Rats, treatment and number	Liver	Muscle	Heart	Stomach	Kidney
Fully fed (12)	**2·3** (∓0·7)	**0·54** (∓0·1)	**0·52** (∓0·17)	**0·21** (∓0·04)	**0·18** (∓0·08)
Starved 24 hours (8)	**0·40** (∓0·15)	**0·35** (∓0·05)	**0·57** (∓0·17)	**0·24** (∓0·02)	**0·18** (∓0·01)
Starved 48 hours (10)	**0·30** (∓0·082)	**0·31** (∓0·12)	**0·52** (∓0·21)	**0·20** (∓0·02)	**0·18** (∓0·07)

B. *Treated with phloridzin.*

Starved 24 hours (10)	**0·23** (∓0·034)	**0·27** (∓0·1)	**0·67** (∓0·17)	**0·19** (∓0·05)	**0·18** (∓0·04)
Starved 48 hours (9)	**0·18** (∓0·07)	**0·19** (∓0·04)	**0·52** (∓0·15)	**0·20** (∓0·06)	**0·16** (∓0·05)

It is clear that 24 hours' and still more 48 hours' starvation greatly reduces liver- and muscle-glycogen. Our results agree with those of Cori and Cori [1928] but differ from those of Barbour *et al.* [1927] who found more glycogen in the liver after 48 than after 24 hours' starvation. Our absolute figures for the starved liver are also higher, but this we are inclined to attribute to the use of 80 % alcohol. We can find no previous determinations of the glycogen of the heart, stomach or kidney of the rat with which to compare our figures. The periods of starvation employed by us make no difference to the glycogen content of the heart, stomach or kidney, a new observation in the latter two organs. As regards the heart Jensen [1902] also found that prolonged starvation of two dogs for 15–17 days did not reduce heart-glycogen, although the latter was found to be very low in one dog after 19 days. This animal, however, was moribund.

Our periods of starvation were not prolonged, but were long enough to show that these three organs are much more tenacious of their glycogen than the liver and skeletal muscle. It seems certain that in the heart, kidney and stomach the formation and breakdown of glycogen differ from these processes

both in the liver and skeletal muscles and are independent of factors which profoundly affect the latter organs.

(2) *The effects of phloridzin.* (*a*) + 24 hours' starvation; (*b*) + 48 hours' starvation; Table II B.

The action of this drug in depleting liver and muscles of glycogen is well-known and has been reported in a voluminous literature. Its action on other organs has been very incompletely studied and the comprehensive reviews of Lusk [1912] and Nash [1927] do not mention it. Junkersdorf [1923] made a few determinations of heart- and kidney-glycogen in starved phloridzinised dogs and found the heart-glycogen raised if anything above the normal level. He does not, however, discuss its significance. 20 mg. (0·2 g. per kg.) of phloridzin was administered subcutaneously in olive oil on three consecutive days. The animals were killed 4–6 hours after the last dose and after 24 or 48 hours' starvation. They became glycosuric after the first dose and soon developed ketonuria. Phloridzin was also administered to a few rats for periods of from 10–14 days with no different result. The blood-sugars of these rats were at low normal levels (0·08–0·1 %).

The averages of our results (Table II B) agree with those of others in showing that phloridzin reduces the glycogen of the liver and muscles slightly below starvation levels. Phloridzin, however, has no effect on the glycogen of the heart, stomach or kidney, which confirms and extends Junkersdorf's experiments.

We do not think the slightly higher glycogen in the phloridzinised rat's heart has any significance. The failure of phloridzin to affect the glycogen of the heart, stomach and kidney is further evidence that the factors controlling the glycogen metabolism of these organs are not the same as those controlling that of the liver or skeletal muscles.

(3) *The effect of thyroid extract.*

Cramer and Krause [1913] first showed that the feeding of thyroid gland reduced the glycogen in the liver of cats and rats to mere traces. This has been confirmed by Kuriyama [1917] and Burn and Marks [1925]. We can find no record of its effect on the glycogen of other organs.

The rats were fed with crushed thyroid tablets (B. and W.) given in melted butter added to their usual diets. In this form the thyroid was well taken. They required 2–3 gr. (0·12–0·19 g.) daily per 100 g. body weight to make them lose weight satisfactorily. Rats therefore require enormous doses compared with rabbits and man. A comparable dose in man would be 2000 gr. daily. The glycogen effects are obtained long before any loss of weight becomes manifest. Table III shows the glycogen distribution in 24 hours' starved hyperthyroid rats, and Table IV that in "fully fed" hyperthyroid animals. The controls are Table II A. Some results require special mention.

(*a*) The hyperthyroid 24 hours' starved liver contains less glycogen than its control and no accumulation of glycogen takes place in the hyperthyroid liver even when "fully fed." These facts were already known and form a

Table III. *Thyroid-fed rats: starved 24 hours.*

Rat	Liver	Muscle	Heart	Stomach	Kidney	Blood-sugar
1	0·16	0·23	0·04*	0·11	0·12	—
2	0·42	0·52	0·17	0·17	0·16	—
3	0·29	Lost	0·18	0·14	0·14	0·118
4	0·22	0·19	0·23	0·09	0·14	0·327
5	0·25	0·21	0·13	0·22	0·14	—
6	0·21	0·12	0·15	0·20	0·14	0·000
7	0·23	0·24	0·24	0·16	0·12	0·056
8	0·18	0·30	0·16	0·14	0·09	0·093
Average	0·25	0·26	0·18	0·15	0·13	—

* Omitted from the average.

Table IV. *Thyroid-fed rats: fully fed.*

Rat	Liver	Muscle	Heart	Stomach	Kidney	Blood-sugar
1	0·26	0·54	0·17	0·39	0·15	0·129
2	0·32	0·53	0·22	0·19	0·17	0·130
3	0·43	0·79	0·58*	0·24	0·24	0·142
4	0·36	0·68	0·22	0·12	0·13	0·126
5	0·21	0·64	0·23	0·14	0·13	0·137
6	0·29	1·00	0·15	0·29	0·18	0·153
7	0·37	1·03	0·12	0·33	0·19	0·139
8	0·35	0·76	0·19	0·24	0·17	0·126
Average	0·32	0·75	0·18	0·24	0·17	—

* Omitted from the average.

strong argument in Cramer's contention that liver-glycogen is not formed directly from ingested carbohydrate, but is a specific secretion of the liver from protein and probably fat. We hope to take this matter up in a further communication.

(*b*) The hyperthyroid 24 hours' starved skeletal muscle contains on the average a little less glycogen than its controls. If this has any significance it may reasonably be explained as the result of the raised basal metabolic rate. The hyperthyroid fully fed muscles on the other hand contain not only as much, but perhaps more glycogen than normal fully fed animals. Bösl [1928] found no reduction in the muscle-glycogen of guinea-pigs injected with thyroxine. It is thus evident that hyperthyroidism has no deleterious effect upon glycogen storage in the muscles and that the glycogen not stored in the hyperthyroid liver may be found in the muscles, due allowance being made for the increased metabolism.

(*c*) The hyperthyroid heart "24 hours' starved" contains very much reduced amounts of glycogen, and these amounts are not increased after food. This effect of thyroid is a very striking one. Is it due to (1) a specific toxic action; (2) overwork; (3) lack of adequate carbohydrate supply? The last is scarcely possible in view of the glycogen storage in these animals' skeletal muscles. We do not think overwork is the reason because adrenaline produces a faster heart rate than thyroid without reducing the glycogen. Also Visscher and Mulder [1930] have recently shown in the heart-lung preparation that work does not reduce heart-glycogen. We are inclined to think therefore that

in this effect thyroid is a specific cardiac toxin. It is interesting to compare the disastrous effects of hyperthyroidism on the human heart.

Histological examination of the hearts of five hyperthyroid rats which died during hot weather in 1930 showed degenerative changes of varying degree, cloudy swelling and poor striation in both auricles and ventricles. In one striation was almost invisible and patches of necrosis with early cellular infiltration were seen. We are indebted to Dr Terence East for this report.

The blood-sugars of the starved hyperthyroid rats show some remarkable variations for which we can offer no explanation. There is no reason, however, to suspect their accuracy.

(*d*) The glycogen in the stomach and kidney of the 24 hours' starved hyperthyroid rats shows some reduction compared with the normal controls. We are inclined to think that the reduction has significance and is produced by the increased metabolism. The glycogen returns to normal after food.

(*e*) *Primary effect on the heart.* After one week (sometimes after 3 days) of thyroid administration the influence of the drug on the glycogen metabolism of the liver and heart has reached its maximum. We have carried out 20 experiments upon animals after from 3 to 6 days' administration to determine whether the heart or the liver was the first to be affected. Table V shows the

Table V. *Animals "fully fed." Early stages of hyperthyroidism.*

	Glycogen %.		
	Liver	Heart	Days' thyroid administration
	1·79	0·19	3
	1·17	0·36	3
	0·68	0·13	4
	1·30	0·15	4
	0·80	0·14	4
	0·86	0·17	4
	0·51	0·08	4
	0·50	0·14	4
	1·40	0·20	4
	0·95	0·20	4
	0·48	0·12	4
	1·70	0·14	6
	1·07	0·32	3
	0·87	0·15	3
	1·90	0·20	6
Average	1·07	0·17	

results excluding (*a*) one whose liver- and heart-glycogen fell within the average deviation from the normal means; (*b*) four animals whose liver- and heart-glycogen fell within the average deviations from the fully fed hyperthyroid means. The table shows that full glycogen depletion of the heart may be obtained when the liver is almost unaffected. No contradictory results were obtained in which the liver was depleted before the heart. The effects of hyperthyroidism emphasise once more that the factors controlling glycogen metabolism may differ from organ to organ.

(4) *The effect of adrenaline.*

This has been made a very special study by Cori and Cori [1928, 1, 2, 3; 1929; 1930] so far as the liver and muscles of the rat are concerned. No other organs have so far as we are aware been investigated. Table VI B shows our experimental results and Table VI A the controls. The mean of our results

Table VI.

Rats, treatment and number	Mean % of glycogen and average deviation from mean						Blood-sugar mg. per 100 cc.
	Liver	Muscle	Heart	Stomach	Kidney	Spleen	
A. Normal (9)	**0·28** (±0·06)	**0·30** (±0·06)	**0·64** (±0·14)	**0·18** (±0·03)	**0·15** (±0·01)*	**0·24** (±0·05)†	**0·085** (±0·011)‡
B. 24 hours' starved; 0·05 mg. adrenaline per 100 g. 2 hours before death (9)	**0·33** (±0·08)	**0·19** (±0·05)	**0·58** (±0·20)	**0·20** (±0·03)	**0·14** (±0·01)	**0·23** (±0·07)†	**0·205** (±0·071)
C. 24 hours' starved; 0·1–0·2 units of insulin per 100 g. (8)	**0·21** (±0·02)	**0·20** (±0·05)	**0·53** (±0·09)	**0·16** (±0·025)	**0·13** (±0·02)	**0·22** (±0·05)†	**0·074** (±0·011)
D. 24 hours' starved; 0·4–3·0 units of pituitary extract 2–3½ hours before death (8)	**0·24** (±0·03)	**0·29** (±0·08)	**0·60** (±0·08)	**0·21** (±0·04)†	**0·13** (±0·01)	**0·18** (±0·025)	**0·082** (±0·015)

* 8 animals. † 7 animals. ‡ 6 animals.

confirms Cori's contention that in the fasting rat adrenaline increases the liver-glycogen and decreases the muscle-glycogen. The glycogen of all the other organs including the heart is not significantly changed by this drug. The mean of the adrenalinised heart is slightly lower, but this is due to two very low results. The remainder are fully as high as the normals. The blood-sugar is raised in all cases by adrenaline, but this has not led to an increased deposition of glycogen in any of the organs.

(5) *The effect of insulin.*

Insulin was given in subconvulsive doses of 0·1–0·2 units per 100 g. and the animals were killed 2 hours later. The results in Table VI C show no appreciable reduction in the glycogen of heart, stomach, kidney or spleen. The liver- and muscle-glycogen are both slightly reduced.

(6) *The effect of pituitrin.*

The results are shown in Table VI D. The glycogen of all the organs, and the blood-sugar, lie very close to the controls, and pituitrin evidently has no effect on glycogen metabolism under these experimental conditions.

(7) *Other organs.*

We have not yet studied the glycogen content of other organs, but have made a few isolated estimations which give the following averages:

	%	Animals
Uterus	0·35	8
Brain	0·15	4
Testicle	0·09	2

General discussion.

The exact chemical nature of glycogen being unknown, any speculation on the possibility of its chemical modification from organ to organ is useless.

Physiologically, however, it is clear that the glycogens of various tissues behave quite differently. This has been accepted as regards liver- and skeletal muscle-glycogen whose products of disintegration, glucose and lactic acid respectively, and whose functions are so entirely different. Isolated observations on heart-glycogen by Cruickshank [1913] and on blood- and general tissue-glycogen by many workers [see Allen, 1913] have shown that it may behave quite differently from liver- and muscle-glycogen in these organs in diabetes. Thus while the diabetic liver may contain only traces of glycogen, and the muscles less than normal, the heart, blood and kidney may contain increased amounts. Our results also show how glycogen metabolism may differ from organ to organ. It is interesting to note the narrow range of glycogen concentration (0·15–0·25 %) in such organs as the kidney, spleen and brain. Also in our experiments various agents have reduced glycogen of the liver, muscle and heart, organs with a higher normal content and perhaps a specialised glycogen function, to similar concentrations. These facts suggest the presence in all organs of a residual non-specific glycogen, which the cells always retain, and which is quite different in physiological behaviour and perhaps chemically from the larger and more variable glycogen content of the liver, muscles and heart. These facts should be taken into account in formulating any complete theory of glycogen metabolism.

Summary.

Table VII contains a summary of the results.

Table VII. *Summary.*

Mean glycogen content of organs of rats.

	Liver	Muscle	Heart	Stomach	Kidney	Spleen	No. of animals
Fully fed	2·30	0·54	0·52	0·21	0·18	0·21	12
Starved 24 hours	0·40	0·35	0·57	0·24	0·18	—	8
Starved 48 hours	0·30	0·31	0·52	0·20	0·18	—	8
Phloridzin. Starved 24 hours	0·23	0·27	0·67	0·19	0·18	—	8
Starved 48 hours	0·18	0·19	0·52	0·20	0·16	—	8
Thyroid. Starved 24 hours	0·25	0·26	0·18	0·15	0·13	—	8
Fully fed	0·32	0·75	0·18	0·24	0·17	0·21	8
3–6 days thyroid	1·07	—	0·17	—	—	—	15
New controls. Starved 24 hours	0·28	0·30	0·64	0·18	0·15	0·23	9
Adrenaline. Starved 24 hours	0·33	0·19	0·56	0·20	0·14	0·23	9
Insulin. Starved 24 hours	0·21	0·20	0·53	0·16	0·13	0·22	8
Pituitrin	0·24	0·29	0·60	0·21	0·13	0·18	8

1. Starvation does not reduce the glycogen of the heart, kidney or stomach.

2. Phloridzin reduces the glycogen of the liver and muscles slightly below starvation level, but has no effect on that of the heart, stomach or kidney.

3. Thyroid extract by mouth reduces very greatly the glycogen of the heart. The hyperthyroid heart is unable to replenish its glycogen stores even after high carbohydrate feeding. After 24 hours' starvation the hyperthyroid liver contains only slightly less glycogen than the control, but has completely

lost the normal ability to store glycogen after carbohydrate food. Th thyroid 24 hours' starved muscle contains slightly less glycogen t normal, but storage is quite normal after carbohydrate ingestion.

The heart-glycogen is the first to be depleted by thyroid[1].

4. Adrenaline increases the liver-glycogen and decreases the muscle-glycogen of starved animals. It has no effect on the glycogen of other organs.

5. Insulin has no effect on the glycogen of heart, stomach or kidney. It reduces liver- and muscle-glycogen slightly.

6. Pituitrin has no effect on the glycogen of the fasting rat.

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[1] *Note added* 14. iv. 31. While this paper was in the Press, we have seen a recent article confirming this by Abderhalden and Wertheimer (1930), *Z. ges. exp. Med.* **72**, 472.

LXVII. METABOLISM IN SCURVY.
III. THE ABSORPTION AND RETENTION OF CALCIUM AND PHOSPHORUS BY GUINEA-PIGS.

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In the last communication of this series, in which the mode of action of the antiscorbutic factor on the animal organism is being studied, it was shown that the pathological changes in the guinea-pig which form the syndrome of scurvy were not preceded by any disturbance in the absorption or in the retention of nitrogen [Shipp and Zilva, 1928]. This report deals with the metabolism of calcium and phosphorus by normal and scorbutic guinea-pigs.

It has already been pointed out by Shipp and Zilva that, since the macroscopic and, more so, the microscopic lesions of scurvy are observed soon after guinea-pigs are placed on a scorbutic diet, any abnormality in the metabolic functions in these animals which may be associated with the production of the disease ought to become manifest in the early stages of the experiment at a time when the animals are consuming adequate quantities of the diet. Changes which take place in the metabolism in later stages when the disease is severe and the animals are declining owing to insufficient consumption of food cannot be considered specific of scurvy. As microscopic scorbutic lesions can be established approximately 8 days after the guinea-pigs have been placed on the scorbutic diet [Zilva and Wells, 1919], it is evident that if the onset of scurvy be subsequent to any specific metabolic disturbance, this latter change will also develop fairly early. Furthermore, its manifestation may not be gradual but rapid, a circumstance which makes it advisable and even necessary to observe the daily intake and output of the elements studied, in order to establish whether, if such disturbance takes place, it precedes the development of the disease or is a consequence of it. The experimental scheme adopted was, therefore, similar to that previously employed by Shipp and Zilva in which the above precautions were taken and in which the retention and absorption of the two elements were also studied up to an advanced stage of experimental scurvy. In addition the calcium and phosphorus content of the blood of scorbutic guinea-pigs at various stages of the disease and of normal guinea-pigs was determined.

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Experimental.

Technique.

The experimental procedure adopted in this investigation was the same as that used by Shipp and Zilva [1928]. The following minor modifications were, however, introduced to suit the requirements of the present work. (1) Instead of preparing a stock solution of milk, sufficient for several days, about 5 g. of dried milk powder were weighed out each day, dissolved in 15 cc. of distilled water and dosed to each guinea-pig. In addition 25 cc. of distilled water which was also offered daily was usually consumed. (2) No acid was placed in the urine collecting vessel, but the cage, funnel, trap and receiving jars were rinsed out each morning with 0·5 *N* HCl, after the careful removal of all faeces, and the washings added to the collected urine.

The calcium in the urine, faeces and blood was estimated by the Kramer and Tisdal method [1921] and the phosphorus by Martland and Robison's modification [1924] of the Briggs [1922] method.

Discussion of results.

The retention and absorption of calcium and phosphorus by protected and scorbutic guinea-pigs.

Exp. 1. This experiment (Table I) was carried out with the view of studying the metabolism of calcium and phosphorus in the protected guinea-pig and, except for the longer duration, it is comparable with the preliminary periods of Exp. 2 when the animals were fed on a diet of basal mixture, autoclaved milk and 5 cc. of decitrated lemon juice daily (Period 1, Tables II and III). The consumption of the basal diet during these preliminary periods was rather below that consumed by guinea-pigs kept in ordinary cages under similar conditions, and, as would be expected, the growth of these animals was less than normal. Apart from this the animals appeared perfectly healthy and active. The average total intake of calcium and phosphorus varied in the three cases and it will be seen that there is a fairly marked variation in the degree of absorption and retention of both elements.

Exp. 2. In this experiment (Guinea-pig 6, Table II) the metabolism of calcium and phosphorus was followed from day to day, both during the preliminary and scorbutic periods. In the preliminary period, although the animal did not show a net gain in weight its consumption of food and general condition of health were those of a protected guinea-pig. In the second period when the administration of decitrated lemon juice was stopped the average excretion and balance were higher than in the preceding period. The urinary calcium was also rather higher than in the first period, but the phosphorus excreted by the kidneys remained of the same order.

Table I. *Guinea-pig No. 9.*

Date Feb. 1929	Weight of animal g.	Basal mixture consumed g.	Total intake of phosphorus mg.	Total intake of calcium mg.	Urinary phosphorus mg.	Faecal phosphorus mg.	Total output of phosphorus mg.	Absorbed phosphorus mg.	Urinary calcium mg.	Faecal calcium mg.	Total output of calcium mg.	Absorbed calcium mg.	Retained phosphorus mg.	Retained calcium mg.	P retained / P intake	P retained / P absorbed	Ca retained / Ca intake	Ca retained / Ca absorbed	Ca retained / P retained
Period 1. 13 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice *per diem*:																			
5	280	12·28	175·9	259·1	66·2	55·3	121·5	120·6	66·1	63·2	129·3	195·9	+ 54·4	+129·8	0·3093	0·4511	0·5013	0·6626	2·368
6	277	13·10	177·1	314·0	86·7	33·0	119·7	144·1	96·9	29·5	126·4	284·5	+ 57·4	+187·6	0·3241	0·3983	0·5973	0·6606	3·268
7	279	14·69	201·9	261·4	81·9	54·2	136·1	147·7	100·3	52·7	153·0	208·7	+ 65·8	+108·4	0·3259	0·4452	0·4148	0·5194	1·647
8	283	16·88	222·8	299·9	55·8	57·3	113·1	165·5	116·0	65·3	181·3	234·6	+109·7	+118·6	0·4925	0·6634	0·3956	0·5058	1·081
9	282	14·42	205·5	248·8	86·0	81·5	167·5	124·0	97·6	88·8	186·4	160·0	+ 38·0	+ 62·4	0·1807	0·3065	0·2502	0·3900	1·643
10	280	13·57	186·2	260·3	93·5	69·4	162·9	116·8	120·4	75·4	195·8	184·9	+ 23·3	+ 64·5	0·1251	0·1995	0·2478	0·3489	2·769
11	275	18·16	233·7	311·2	102·1	65·0	167·1	168·7	132·6	72·1	204·7	239·1	+ 66·6	+106·5	0·2849	0·3949	0·3423	0·4454	1·599
12	282	9·16	128·4	181·7	75·8	28·3	104·1	100·1	115·2	41·0	156·2	140·7	+ 24·3	+ 25·5	0·1888	0·2428	0·1402	0·1812	1·049
13	280	14·00	195·1	244·6	86·4	35·8	122·2	159·3	104·1	57·4	161·5	187·2	+ 72·9	+ 83·1	0·3741	0·4576	0·3397	0·4439	1·140
14	285	13·16	158·4	235·5	66·0	38·8	104·8	119·6	98·9	57·9	156·8	177·6	+ 53·6	+ 78·7	0·3385	0·4481	0·3345	0·4431	1·469
15	288	14·07	191·9	245·8	76·9	37·8	114·7	154·1	108·0	58·9	166·9	186·9	+ 77·2	+ 78·9	0·4024	0·5009	0·3214	0·4222	1·022
16	285	13·70	176·4	232·8	89·3	85·2	174·5	91·2	118·7	105·5	224·2	127·3	+ 1·9	+ 8·6	0·0107	0·0208	0·0369	0·0676	4·526
17	280	15·26	174·1	256·5	81·9	57·3	139·2	116·8	121·7	79·8	201·5	176·7	+ 34·8	+ 55·0	0·1999	0·2980	0·2141	0·3113	1·581
Total		182·45	2427·4	3351·6	1048·5	698·9	1747·4	1728·5	1396·5	847·5	2244·0	2504·1	+680·0	+1107·6	0·2805	0·3928	0·3274	0·4424	1·63
Average			186·7	257·8	80·6	53·7	134·5	132·9	107·4	65·2	172·6	192·6	52·3	85·2					

"Absorbed" phosphorus (or calcium) = total intake of phosphorus (or calcium) less faecal phosphorus (or calcium).
"Retained" phosphorus (or calcium) = total intake of phosphorus (or calcium) less total output of phosphorus (or calcium).

Table II. *Guinea-pig No. 6.*

Date 1929 Mar.	Weight of animal g.	Basal mixture consumed g.	Total intake of phosphorus mg.	Total intake of calcium mg.	Urinary phosphorus mg.	Faecal phosphorus mg.	Total output of phosphorus mg.	Absorbed phosphorus mg.	Urinary calcium mg.	Faecal calcium mg.	Total output of calcium mg.	Absorbed calcium mg.	Retained phosphorus mg.	Retained calcium mg.	P retained / P intake	P retained / P absorbed	Ca retained / Ca intake	Ca retained / Ca absorbed	Ca retained / P retained
Period 1. 8 days on basal diet and autoclaved milk and 5 cc. decitrated lemon juice *per diem*:																			
14	337	20·01	240·7	354·4	90·6	66·4	157·0	174·3	131·7	94·5	226·2	259·9	+ 83·7	+128·2	0·3477	0·4803	0·3617	0·4933	1·532
15	340	18·26	233·3	355·3	99·8	108·7	208·5	124·6	121·7	126·1	247·8	229·2	+ 24·8	+107·5	0·1064	0·1581	0·3026	0·4691	4·335
16	330	20·02	246·8	351·3	85·8	105·6	191·4	141·2	115·7	123·4	239·1	227·9	+ 55·4	+112·2	0·2245	0·3923	0·3203	0·4923	2·025
17	332	18·77	231·9	341·5	83·9	110·4	194·3	121·5	105·4	127·1	232·5	214·4	+ 37·6	+109·0	0·1623	0·3102	0·3192	0·5084	2·898
18	337	13·85	215·1	302·5	62·9	72·4	135·3	142·7	82·2	82·1	164·3	220·4	+ 79·8	+138·2	0·3710	0·5593	0·3629	0·6270	1·376
19	342	8·28	122·9	183·0	41·0	120·7	161·7	2·2	37·9	130·0	167·9	53·0	− 38·8	+ 15·1	—	—	0·0824	0·2830	—
20	340	8·79	136·3	190·2	48·8	57·3	106·1	79·0	46·4	88·6	135·0	101·6	+ 30·2	+ 55·2	0·2216	0·3823	0·2970	0·5421	1·828
21	335	16·95	218·3	316·9	55·2	75·3	130·5	143·0	98·4	97·7	196·1	219·2	+ 87·8	+120·8	0·4038	0·6296	0·3812	0·5511	—
Total		124·93	1645·3	2395·1	568·0	716·8	1284·8	928·5	739·4	869·5	1608·9	1525·6	+360·7	+786·2	0·2193	0·3886	0·3282	0·5159	2·108
Average		15·62	205·7	299·4	71·0	89·6	160·6	116·1	92·4	108·7	201·1	190·7	+ 45·1	+ 98·3					
Period 2. 5 days on basal diet and autoclaved milk:																			
22	335	20·04	250·7	353·3	71·7	114·9	186·6	135·8	127·9	129·7	257·6	223·6	+ 64·1	+ 95·7	0·2558	0·4721	0·2708	0·4280	1·493
23	343	21·33	257·6	378·1	76·1	184·2	260·3	73·4	114·9	186·5	301·4	191·6	− 2·7	+ 76·7	—	—	0·2029	0·4004	—
24	340	20·64	252·4	359·9	97·6	144·4	242·0	108·0	127·8	146·2	274·0	213·7	+ 10·4	+ 85·9	0·0412	0·0943	0·2386	0·4020	8·262
25	345	21·83	288·2	403·9	79·7	83·2	162·9	205·0	124·1	127·4	251·5	276·5	+125·3	+152·4	0·4347	0·6110	0·3772	0·5511	1·334
26	348	22·29	285·8	395·0	69·8	99·7	169·5	186·1	107·6	184·0	291·6	211·0	+116·3	+103·4	0·4070	0·6250	0·2617	0·4900	0·889
Total		106·13	1334·7	1890·2	394·9	626·4	1021·3	708·3	602·3	773·8	1376·1	1116·4	+313·4	+514·1	0·2336	0·4432	0·2658	0·4606	1·640
Average		21·23	266·9	378·0	79·0	125·3	204·3	141·7	120·5	154·8	255·2	223·3	+ 62·7	+102·8					
Period 3. 5 days on basal diet and autoclaved milk:																			
27	352	21·69	361·8	376·3	69·2	145·2	214·4	216·6	105·3	140·4	245·7	235·9	+147·4	+130·6	0·4074	0·6969	0·3469	0·5530	0·886
28	354	18·84	233·8	369·2	65·6	49·3	114·9	184·5	106·9	108·3	215·2	260·9	+118·9	+154·0	0·5098	0·6446	0·4172	0·5902	1·295
29	360	16·83	211·4	354·9	67·9	128·0	195·9	83·4	108·8	93·9	202·7	261·0	+ 15·5	+152·2	0·0733	0·1858	0·4288	0·5968	9·819
30	362	19·93	238·1	336·4	70·9	100·6	171·5	137·5	119·9	99·1	219·0	237·3	+ 66·6	+117·4	0·2803	0·4844	0·3920	0·4948	1·763
31	372	19·58	244·4	350·5	84·7	121·2	205·9	123·2	111·8	149·8	261·6	200·7	+ 38·5	+ 88·9	0·1575	0·3125	0·2536	0·4430	2·310
Total		96·87	1289·5	1787·3	358·3	544·3	902·6	745·2	552·7	591·5	1144·2	1195·8	+386·9	+643·1	0·2999	0·5192	0·3599	0·5378	1·663
Average		19·37	257·9	359·5	71·7	108·9	180·5	149·0	110·5	108·3	228·8	239·2	+ 77·4	+128·6					

Period 4. 5 days on basal diet and autoclaved milk:

Apr.																		
1	370	19·08	239·4	339·6	87·5	64·9	152·4	174·5	124·3	137·6	261·9	202·0	+ 87·0	+ 77·7	0·3631	0·4986	0·2288	0·3846	0·893
2	363	18·29	233·6	346·1	84·3	64·6	148·9	169·0	116·0	142·2	258·2	203·9	+ 84·7	+ 87·9	0·3626	0·5012	0·2533	0·4311	1·038
3	357	16·21	214·6	330·2	82·6	54·8	137·4	159·8	129·3	100·3	229·6	229·9	+ 77·2	+100·6	0·3597	0·4831	0·3045	0·4375	1·303
4	355	17·03	220·9	339·2	83·7	44·5	128·2	176·4	98·6	99·4	198·0	239·8	+ 92·7	+140·2	0·4198	0·5255	0·4133	0·5992	1·512
5	360	17·21	206·4	302·1	84·4	65·6	150·0	140·8	93·2	115·9	209·1	186·2	+ 56·4	+ 93·0	0·2797	0·4006	0·3079	0·4995	1·649
Total		87·82	1114·9	1657·2	422·5	294·4	716·9	820·5	561·4	595·4	1156·8	1061·8	+398·0	+500·4	0·3570	0·4850	0·3020	0·4711	1·258
Average		17·56	223·0	331·4	84·5	58·9	143·4	164·1	112·3	119·1	231·4	212·4	+ 79·6	+100·1					

Period 5. 5 days on basal diet and autoclaved milk:

6	352	10·00	113·3	236·2	76·2	83·8	160·0	29·5	78·1	96·7	174·8	139·5	− 46·7	+ 61·4	—	—	0·2599	0·4391	—
7	340	7·64	98·2	169·8	66·1	45·9	112·0	52·3	68·1	69·8	137·9	100·0	− 13·8	+ 31·9	—	—	0·2364	0·3190	—
8	330	7·35	79·7	134·7	69·8	45·2	115·0	34·5	65·1	57·2	122·3	77·5	− 35·3	+ 12·4	—	—	0·0920	0·1600	—
9	337	8·22	116·4	174·1	65·6	38·5	104·1	77·9	72·8	63·1	135·9	111·0	+ 12·3	+ 38·2	0·0946	0·1579	0·2195	0·3442	3·106
10	332	5·11	81·1	121·9	49·5	37·2	86·7	43·9	55·3	68·6	123·9	53·3	− 5·6	− 2·0	—	—	—	—	—
Total		38·32	488·7	836·7	327·2	250·6	577·8	238·1	339·4	355·4	694·8	481·3	− 89·1	+141·9	—	—	0·1692	0·2944	—
Average		7·66	97·7	187·3	65·4	50·1	115·8	47·6	67·9	71·1	139·0	96·3	− 17·8	+ 28·4					

Period 6. 4 days on basal diet, autoclaved milk and 5 cc. decitrated lemon juice:

11	322	5·88	86·5	138·4	47·0	39·8	86·8	46·7	58·9	68·9	127·8	69·5	− 0·3	+ 10·6	—	—	0·0766	0·1525	—
12	315	7·21	113·1	158·3	44·8	33·7	78·5	79·4	59·6	62·4	122·0	95·9	+ 34·6	+ 36·3	0·3263	0·4358	0·2293	0·3785	1·047
13	302	5·00	76·8	153·3	52·3	47·5	99·8	29·3	92·5	76·1	168·6	77·2	− 23·0	− 15·3	—	—	—	—	—
14	290	4·82	75·4	140·4	41·3	47·5	88·8	27·9	87·6	65·5	153·1	74·9	− 13·4	− 12·7	—	—	—	—	—
Total		22·91	351·8	590·4	185·4	168·5	353·9	183·3	298·6	272·9	571·5	317·5	− 2·1	+ 18·9	—	—	0·0322	0·0598	—
Average		5·73	88·0	147·6	46·4	44·6	88·5	45·8	74·7	68·2	142·9	79·4	− 0·5	+ 4·7					

Table III. *Guinea-pig No. 11.*

Date 1929 May	Weight of animal g.	Basal mixture consumed g.	Total intake of phosphorus mg.	Total intake of calcium mg.	Urinary phosphorus mg.	Faecal phosphorus mg.	Absorbed phosphorus mg.	Urinary calcium mg.	Faecal calcium mg.	Absorbed calcium mg.	Retained phosphorus mg.	Retained calcium mg.	P retained / P intake	P retained / P absorbed	Ca retained / Ca intake	Ca retained / Ca absorbed	Ca retained / P retained
Period 1. 7 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice:																	
6	300	14·47	196·1	263·3	73·5	58·1	138·0	119·0	123·6	139·7	+64·5	+ 20·7	0·3290	0·4774	0·07861	0·1480	0·3201
7	300	14·08	202·0	293·8	72·9	80·8	121·2	112·3	141·4	152·4	+48·4	+ 40·1	0·2394	0·3996	0·1365	0·2523	0·8285
8	297	17·33	223·2	323·8	74·6	82·2	141·0	134·9	122·9	200·9	+66·4	+ 56·0	0·2975	0·4743	0·1730	0·2786	0·8433
9	318	18·91	225·3	357·1	82·3	98·1	127·2	131·5	157·7	199·4	+44·8	+ 67·9	0·1988	0·3572	0·1897	0·3405	1·4950
10	321	17·35	240·7	337·9	77·5	101·6	139·1	146·6	145·9	192·0	+61·6	+ 45·4	0·2560	0·4439	0·1344	0·2634	0·7528
11	323	17·08	246·7	341·4	83·3	102·8	143·9	131·4	159·5	181·9	+60·5	+ 50·5	0·2453	0·4206	0·1479	0·2776	0·8347
12	320	16·53	229·2	338·2	92·4	84·9	144·3	144·8	133·2	205·0	+51·8	+ 60·2	0·2260	0·3589	0·1782	0·2936	1·1620
Total		115·75	1563·2	2255·5	556·4	608·5	954·7	920·5	984·2	1271·3	+398·0	+350·8	0·2554	0·4172	0·1511	0·2681	0·8559
Average		16·54	223·2	322·2	79·5	86·9	136·4	131·5	126·3	181·6	+56·7	+ 50·1					
Period 2. 5 days on basal mixture and autoclaved milk, no antiscorbutic:																	
13	322	18·95	255·9	365·5	74·6	139·8	116·1	112·1	177·1	188·4	+41·5	+ 76·3	0·1622	0·3574	0·2092	0·4050	1·8390
14	320	15·35	233·7	316·6	74·6	96·7	137·0	117·8	132·8	183·8	+62·4	+ 66·0	0·2670	0·4565	0·2084	0·3590	1·0580
15	320	14·43	196·9	281·5	53·6	135·9	61·0	88·4	167·2	194·3	+ 7·4	+ 25·9	0·0376	0·1211	0·0944	0·1332	3·4990
16	310	19·02	227·1	332·0	62·5	138·9	88·2	114·0	160·3	171·7	+25·7	+ 57·7	0·1132	0·2914	0·1738	0·3359	2·2460
17	310	19·01	244·1	336·4	82·3	154·3	89·8	121·3	177·2	169·2	+ 7·5	+ 37·9	0·0307	0·0816	0·1127	0·2240	5·0530
Total		86·79	1157·7	1632·0	347·6	665·6	492·1	553·6	814·6	817·4	+144·5	+263·8	0·1248	0·2936	0·1615	0·3228	1·8220
Average		17·36	231·5	326·5	69·5	133·1	98·4	110·7	162·9	163·5	+28·9	+ 52·8					
Period 3. 6 days on basal mixture and autoclaved milk, no antiscorbutic:																	
18	313	17·99	247·6	338·5	72·6	134·6	113·0	115·2	157·0	181·5	+40·4	+ 65·3	0·1631	0·3576	0·1930	0·3597	1·6160
19	310	17·96	223·0	310·8	73·8	135·0	88·0	115·0	155·0	155·8	+14·2	+ 41·8	0·0637	0·1612	0·1345	0·2683	2·9400
20	312	19·32	240·6	326·4	76·9	123·8	116·8	122·4	143·9	182·5	+39·9	+ 60·1	0·1533	0·3159	0·1841	0·3294	1·6290
21	313	18·30	224·5	304·5	73·8	124·6	99·9	128·9	133·0	171·5	+26·1	+ 42·6	0·1160	0·2612	0·1351	0·2456	1·6100
22	315	17·72	213·2	318·1	66·9	130·1	83·1	121·6	158·0	160·1	+16·2	+ 38·5	0·0760	0·1949	0·1211	0·2400	2·3770
23	320	16·72	203·5	335·1	62·9	106·0	97·5	103·2	137·1	198·0	+34·6	+ 94·8	0·1700	0·3557	0·2830	0·4787	2·7390
Total		108·01	1352·4	1933·4	426·9	754·1	598·3	706·3	884·0	1049·4	+171·4	+343·1	0·1268	0·2865	0·1776	0·3271	2·0020
Average		14·00	225·4	322·2	71·2	125·7	99·7	117·7	148·7	174·9	+28·6	+ 57·2					

Average		14·00	225·4	322·2	71·2	125·7	99·7	117·7	148·7	174·9	+28·6	+ 57·2				

Period 4. 5 days on basal mixture and autoclaved milk, no antiscorbutic:

24	323	18·40	210·3	344·4	73·0	142·4	67·9	135·5	171·0	173·4	− 5·1	+ 37·9	—	—	0·1100	0·2186	—
25	324	14·63	166·7	283·0	64·7	130·5	36·2	107·6	158·2	124·8	−28·5	+ 17·2	—	—	0·0765	0·1378	—
26	327	14·73	167·6	266·6	71·2	102·0	65·6	116·8	114·7	151·9	− 5·6	+ 35·1	—	—	0·1316	0·2311	—
27	328	12·09	144·1	212·7	63·1	100·9	43·2	85·5	120·2	92·5	−19·9	+ 7·0	—	—	0·3291	0·0756	—
28	320	10·36	132·6	234·0	60·2	75·7	56·9	92·5	84·3	149·7	− 4·3	+ 57·1	—	—	0·2440	0·3814	—
Total		70·21	821·3	1340·7	332·2	551·5	269·8	537·9	648·4	692·3	−63·4	+154·3	—	—	0·1156	0·2229	—
Average		14·04	164·3	268·1	66·4	110·3	54·0	107·6	129·7	138·5	−12·7	+ 30·9					

Period 5. 5 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice:

29	322	11·45	163·5	234·6	69·0	79·6	83·9	93·5	73·6	161·0	+13·8	+ 67·6	0·0838	0·1632	0·2822	0·2595	4·8210
30	317	12·14	178·4	251·0	74·5	76·7	101·7	108·3	99·9	151·1	+26·2	+ 42·8	0·1469	0·2577	0·1705	0·2832	1·6330
31	312	14·38	200·6	278·4	68·5	83·9	116·7	97·9	100·8	177·6	+51·2	+ 79·7	0·2496	0·4377	0·2864	0·4477	1·5570
June																	
1	318	15·11	203·0	286·1	67·6	122·1	80·9	101·1	150·3	135·8	+13·4	+ 34·7	0·0660	0·1657	0·1213	0·2555	2·5890
2	313	16·06	221·8	303·3	66·7	86·8	135·0	99·6	115·9	187·4	+68·3	+ 87·7	0·3080	0·5059	0·2892	0·4651	1·2770
Total		69·14	967·3	1353·4	346·3	449·1	518·2	500·4	540·5	812·9	+172·9	+312·5	0·1787	0·3336	0·2312	0·3844	1·8070
Average		13·83	193·5	270·7	69·3	89·8	103·6	100·1	108·1	162·6	+ 34·6	+ 62·5					

Period 6. 4 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice:

3	322	16·88	222·5	352·2	71·7	91·7	130·8	100·9	124·4	227·8	+59·2	+ 99·9	0·2661	0·4307	0·2844	0·4386	1·6880
4	321	15·29	199·4	280·5	92·5	103·0	96·4	103·6	123·4	157·1	+ 3·9	+ 53·5	0·1956	0·4049	0·1907	0·3406	13·7200
5	321	17·41	228·9	333·2	61·9	119·9	109·0	99·0	122·6	210·6	+47·0	+111·6	0·2053	0·4312	0·3273	0·5299	2·3740
6	325	18·15	239·5	354·8	64·1	108·2	131·3	99·8	111·9	242·9	+67·2	+143·2	0·2806	0·5118	0·4035	0·5895	2·1250
Total		67·73	890·3	1320·7	290·2	422·8	467·5	403·3	482·3	838·4	+177·3	+408·2	0·1992	0·3793	0·3154	0·5029	2·3150
Average		16·93	222·6	330·2	72·5	105·7	116·9	100·8	120·6	209·6	+44·3	+102·1					

However, as far as one can judge from these figures and those of the following experiment, no striking deviation from the normal metabolism of calcium and phosphorus takes place during the first 10 days on a scorbutic diet. During the following two scorbutic periods (4 and 5) the intake of food diminished and the balance eventually fell in consequence, that of phosphorus actually becoming negative. The faecal phosphorus diminished in both periods whilst the calcium did so only during period 5. There was a decided drop in the urinary calcium and phosphorus during this period. These changes certainly show a deviation from the normal, but they are, presumably, not connected with the onset of scurvy but are the effect of a general metabolic disturbance produced by the disease. In the last period decitrated lemon juice was again given to the animal but, as may be seen from the figures, the disturbance in the metabolism was too advanced to be rectified during the 4 days of treatment.

If we now turn our attention to guinea-pig 11 (Table III) we see a picture similar to that presented by guinea-pig 6. In view of the observations previously made here to the contrary one cannot attach much importance to the slight average rise in the faecal, and fall in the urinary, calcium during periods 2 and 3. On the other hand, as in the case of guinea-pig 6, there was a marked rise in the faecal phosphorus during these periods. This observation was considered at the time the more noteworthy since there was not even an increased intake of this element during these periods. It will be seen that in the case of guinea-pig 11 there were only three scorbutic periods (15 days) and consequently the return to normal metabolism took place shortly after resuming the administration of the antiscorbutic dose.

Exp. 3. It was seen in Exp. 2 that a rise in the faecal excretion of phosphorus by both guinea-pigs occurred during the first two scorbutic periods whilst the calcium was not affected. In guinea-pig 6 this could to some extent be explained by the increased food intake during the early days on the scorbutic diet. Such an increased consumption, however, did not, as already mentioned, occur in the case of guinea-pig 11. It is evident that if a raised faecal excretion of phosphorus were characteristic of the onset of scurvy it should be possible to repeat it without difficulty, since the production of experimental scurvy in young guinea-pigs is marked by little individual variation. The daily intake and faecal excretion of phosphorus were therefore studied in three guinea-pigs, but no rise in the faecal phosphorus could be observed. For the sake of economy of space the averages of the various periods are given in Tables IV, V and VI. It will be seen that in the case of guinea-pig 14 (Table IV) there is even a somewhat smaller ratio of faecal to consumed phosphorus during the first two scorbutic periods than in the preceding preliminary periods, whilst in guinea-pigs 17 and 19 there is no appreciable change to be observed in this ratio. It seems, therefore, highly improbable that an increased faecal excretion of phosphorus is primarily associated with the onset of scurvy in the guinea-pig and the rise observed in

Table IV. *Guinea-pig No. 14.*

Period	Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P / Intake of P
1 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+15	831·30	494·21	166·26	98·84	0·5946
2 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	− 3	590·40	341·40	147·60	85·35	0·5782
3 5 days on basal diet and autoclaved milk	0	739·31	349·11	147·86	69·82	0·4720
4 4 days on basal diet and autoclaved milk	+16	871·07	414·75	217·77	103·69	0·4760
5 4 days on basal diet and autoclaved milk	+ 2	809·78	379·81	202·45	94·95	0·4689
6 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+15	1075·09	624·68	215·02	124·94	0·5812
7 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+10	921·59	495·30	230·40	123·83	0·5379
8 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+10	957·26	604·80	239·32	151·20	0·6319
9 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	0	930·42	499·81	232·61	124·95	0·5374

Table V. *Guinea-pig No. 19.*

Period	Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P / Intake of P
1 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+ 2	970·11	489·47	194·02	97·89	0·5046
2 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+10	883·41	397·28	220·85	99·32	0·4504
3 5 days on basal diet and autoclaved milk	+23	925·60	454·50	185·12	90·90	0·4911
4 4 days on basal diet and autoclaved milk	+10	990·79	519·30	247·70	129·83	0·5242
5 4 days on basal diet and autoclaved milk	0	724·33	365·78	181·08	91·45	0·5049
6 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+13	1028·25	544·71	205·65	108·94	0·5297
7 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+ 7	981·77	447·61	245·44	111·90	0·4557
8 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+20	1074·75	582·70	268·69	145·68	0·5423
9 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+10	1033·62	535·50	258·41	133·88	0·5177

Table VI. *Guinea-pig No. 17.*

Period	Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P / Intake of P
1 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	− 8	778·21	397·09	155·64	79·42	0·5104
2 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+ 3	814·00	423·83	203·50	105·96	0·5206
3 4 days on basal diet and autoclaved milk	+ 8	725·54	381·91	181·39	95·48	0·5264
4 4 days on basal diet and autoclaved milk	+12	861·93	455·91	215·48	113·98	0·5291
5 4 days on basal diet and autoclaved milk	− 8	731·71	379·07	182·93	94·77	0·5180
6 5 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+ 5	883·30	413·55	176·66	82·71	0·4680
7 4 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+17	800·44	393·47	200·11	98·37	0·4919
8 6 days on basal diet, autoclaved milk and 5 cc. of decitrated lemon juice *per diem*	+16	1407·86	571·13	234·64	95·19	0·4057

Exp. 2 must have been due to a cause other than the consumption of the scorbutic diet.

The calcium and phosphorus content of the blood of protected and scorbutic guinea-pigs.

Before proceeding to study the calcium and phosphorus content of the blood it was essential to ascertain how the composition of the blood in this respect was affected by the time elapsing between the animal's last meal and the collection of the blood for analytical purposes. In the case of a diet offered *ad lib.* it is impossible to know when the animal has its last feed and therefore if the calcium and phosphorus of the blood were influenced by the time of consumption of the last meal it would obviously vitiate any comparative results unless precautions were taken.

Table VII.

Guinea-pigs nos.	Weight g.	Diet	Remarks	Phosphorus whole blood mg./100 cc.
22	375	Oats, bran and cabbage *ad lib.*	Starved 18 hrs. prior to bleeding	36·35
36	380	,,	,,	31·62
27, 28	247, 250	,,	,,	34·61
39, 38	275, 270	Basal diet, *ad lib.* 40 cc. milk and 5 cc. decitrated lemon juice	,,	41·36
31, 37	287, 280	,,	Not starved	39·83
30, 29	275, 300	,,	,,	47·92

All animals bled by severing the jugular vein and carotid artery after anaesthesia.

Exp. 4. All the details of this experiment are summarised in Tables VII and VIII. The results make it plain that although there is a considerable individual variation there is no indication that the blood-calcium and blood-phosphorus of guinea-pigs fed on the basal diet and receiving their anti-scorbutic in the form of decitrated lemon juice are influenced by the length of the interval between the last meal and the collection of the blood.

Table VIII.

Guinea-pigs nos.	Weight g.	Diet	Remarks	Calcium whole blood mg./100 cc.
168, 178	280, 235	Basal, 40 cc. milk and 5 cc. decitrated lemon juice	Starved 18 hrs. Bled 1¾ hrs. after giving food	9·149
175, 174	275, 277	,,	,,	9·897
169, 164	247, 290	,,	Starved 18 hrs. Bled 5 hrs. after giving food	9·623
173, 163	240, 280	,,	,,	8·292
165, 161	245, 250	,,	Starved 18 hrs. prior to bleeding	9·942
172, 170	245, 265	,,	,,	9·813
160, 166	250, 255	,,	Not starved	9·806
177, 179	237, 255	,,	,,	11·44
181, 185	260, 285	,,	,,	8·515
189, 190	280, 265	,,	,,	9·811
202, 203	275, 330	Basal, 40 cc. milk and 10 cc. decitrated lemon juice	,,	8·974
198, 193	295, 315	,,	,,	8·855

All animals killed by cutting the jugular vein and carotid artery without anaesthesia.

Exp. 5. A number of guinea-pigs were then fed on the basal diet and 5 cc. of decitrated lemon juice *per diem* for about 16 days. Some were then killed by bleeding whilst others were kept on the same basal diet but without the antiscorbutic dose for further periods of 5, 8, 10, 15 and 20 days respectively before being bled. As will be seen from Table IX no change in the

Table IX.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Phosphorus whole blood mg./100 cc.
29, 30	275, 300	Basal, autoclaved milk and 5 cc. decitrated lemon juice	0	47·92
31, 37	280, 287	,,	0	39·85
38, 39	275, 300	,,	0	41·36
40, 49	305, 325	Basal and milk	5	40·86
50, 51	326, 302	,,	5	44·29
41, 42	282, 252	,,	8	34·50
43, 44	245, 325	,,	10	36·81
53, 55	255, 265	,,	10	41·91
46, 48	215, 230	,,	15	33·38
56	335	,,	15	49·90
45, 47	255, 227	,,	20	37·57
52, 57	299, 318	,,	20	43·19

All animals killed by cutting the jugular vein and carotid artery after anaesthesia.

total phosphorus content of the blood was observed. As before great individual variations exist which cannot be traced to any change in diet.

Exp. 6. In Exps. 4 and 5 the guinea-pigs were anaesthetised prior to being bled. It was therefore decided to determine whether the elimination of the anaesthetic would bring about smaller individual variations in the blood-phosphorus. Table X gives the results of an experiment in which the blood

Table X.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Calcium whole blood mg./100 cc.	Phosphorus whole blood mg./100 cc.
84, 87, 93	230, 280, 250	Basal and 5 cc. decitrated lemon juice	0	13·50	38·10
85, 89, 94	268, 282, 264	,,	0	12·20	36·16
90, 91, 92	277, 303, 282	,,	0	15·37	32·20
83, 86, 88	262, 270, 277	,,	0	15·32	33·95
61	300	,,	0	15·47	—
60	290	,,	0	15·38	—
62, 63	270, 320	Basal only	5	12·59	40·30
74, 75	300, 320	,,	5	11·28	40·60
64, 65	322, 365	,,	10	10·98	43·60
76, 78	305, 320	,,	10	11·22	43·65
67, 68	315, 325	,,	15	10·48	42·00
77, 80	270, 300	,,	15	12·84	36·76
70, 71	325, 315	,,	20	13·90	41·75
81, 82	340, 285	,,	20	14·42	41·55

All animals bled by severing the jugular vein and carotid artery without anaesthesia.

was collected without previously anaesthetising the animal. The variations in the blood-phosphorus were, however, still marked (Table X) but a somewhat lower content of calcium and higher average content of phosphorus were found in the whole blood during the scorbutic period. As the subsequent experiments will show, this was not due to the absence of the antiscorbutic factor.

Exp. 7. The effect of increasing the antiscorbutic dose was next investigated. The animals were given 10 cc. of decitrated lemon juice daily instead of 5 cc. as in the previous experiments. From Table XI it is seen that the

Table XI.

Guinea-pigs nos.	Weight g.	Calcium whole blood mg./100 cc.	Phosphorus whole blood mg./100 cc.
98	315	9·482	37·20
95	315	9·988	40·25
102	332	8·410	40·98
96	320	9·114	42·01
101	325	8·110	41·51
99	322	8·023	37·96
107	285	9·471	—

All animals bled by cutting the jugular vein and carotid artery without anaesthesia.
Diet—basal mixture, autoclaved milk and daily dose of 10 cc. decitrated lemon juice.

blood-phosphorus of guinea-pigs receiving 10 cc. of decitrated lemon juice daily is of the same order as that of the scorbutic animals in Exps. 5 and 6,

(Tables IX and X) whilst the blood-calcium is lower than that of the scorbutic and protected animals previously examined in Exp. 6 (Table X).

That this difference in the blood-calcium is not due to the higher anti-scorbutic dose is seen from the following experiment. A number of guinea-pigs were fed for about two weeks on the basal diet and autoclaved milk, one-half of them receiving 5 cc. and the remainder 10 cc. of decitrated lemon juice *per diem*. The antiscorbutic dose was then discontinued in both cases and the animals were killed after subsisting on the basal diet alone for 5, 8 and 10 days. The results (Tables XII and XIII) also show considerable individual variation but again give no indication of any alteration in the calcium and phosphorus content of the blood which can be traced to the exclusion of vitamin C from the diet.

Table XII.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Calcium whole blood mg./100 cc.
160, 166	259, 255	Basal, autoclaved milk and 5 cc. decitrated lemon juice	0	9·806
177, 179	237, 255	,,	0	11·44
181, 185	260, 285	,,	0	8·515
189, 190	280, 265	,,	0	9·811
202, 203	275, 330	Basal, autoclaved milk and 10 cc. decitrated lemon juice	0	8·974
198, 193	295, 315	,,	0	8·855
183, 186	335, 280	Basal and autoclaved milk	5	8·845
182, 187	270, 307	,,	5	9·770
194, 199	255, 285	,,	5	8·800
195	315	,,	5	9·603
188, 191	340, 295	,,	10	9·705
180	325	,,	10	12·34
197, 201	275, 325	,,	10	9·301
176, 200	240, 260	,,	10	10·11

All animals bled by cutting the jugular vein and carotid artery without anaesthesia.

Table XIII.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorubtic diet	Phosphorus whole blood mg./100 cc.
204, 207	395, 330	Basal and autoclaved milk, 5 cc. decitrated lemon juice	0	34·98
214, 213	340, 310	,,	0	43·17
225, 217	335, 275	,,	0	35·01
226, 228	285, 325	Basal and autoclaved milk, 10 cc. decitrated lemon juice	0	36·16
233, 236	285, 235	,,	0	42·40
234	305	,,	0	35·25
205, 218	245, 365	Basal and milk	5	39·62
211, 215	340, 260	,,	5	36·64
227, 229	260, 375	,,	5	34·10
230, 232	305, 335	,,	8	42·81
242, 243	255, 265	,,	8	37·91
209, 212	405, 300	,,	10	38·39
220, 221	280, 365	,,	10	42·10
239, 237	375, 305	,,	10	36·46
241	315	,,	10	43·65

All animals killed by cutting the jugular vein and carotid artery without anaesthesia.

Conclusion.

This investigation reveals no significant change in calcium and phosphorus metabolism in the early stages of scurvy in guinea-pigs. As in the case of nitrogen [Shipp and Zilva, 1928], the balance becomes negative only when the disease has reached the stage at which there is a general disturbance in the physiological functions of the organism, a change which must be regarded as secondary in character and not specific for the disease. The only investigators who have published a complete balance sheet for protected and scorbutic guinea-pigs are Baumann and Howard [1917], who found a negative phosphorus and calcium balance in adult guinea-pigs (400–500 g.) kept on a scorbutic diet of oats and water for 14 days. On examining their figures, however, it becomes evident that this negative balance was not due to the scorbutic nature of the diet but to the low intake of the elements under discussion, since their animals consumed only 5 mg. of calcium and 30–40 mg. of phosphorus daily and lost 32 % of their body weight in the 14 days on the scorbutic diet. This loss is rather high even for guinea-pigs kept in metabolism cages, and was probably due to an insufficient consumption of food in general.

It is also evident from our investigation that the calcium and phosphorus content of the blood of normal guinea-pigs varies considerably, a fact previously recorded by Edelstein and Schmal [1926] for serum and by Teich [1929] for whole blood of these animals. Only very considerable variations in the blood of animals on a scorbutic diet can therefore be accepted as significant. Like Edelstein and Schmal we observed no such changes. In only one experiment is there any change which appears to be associated with the change of diet (Table X: lower calcium and higher phosphorus in the scorbutic than in the normal period) and in this case it is the figures for the normal animals that are outside the usual range. Euler and Myrbäck [1925] noted a fall in the phosphorus content of the blood of scorbutic animals and suggested that this change might be utilised in the early diagnosis of scurvy in these animals. This conclusion was arrived at by Euler and Myrbäck from the examination of the blood of only two normal and three scorbutic guinea-pigs and it is probable that the difference was due to individual variation and not to the deficient diet.

Summary.

There is no deviation from the normal in the absorption or retention of calcium or of phosphorus before or during the development of scurvy in guinea-pigs. Disturbances in the calcium and phosphorus balances are noted only in the last stages of the experiment when all the functions of the animal organism become deranged by the disease.

The calcium and phosphorus content of the blood of protected and scorbutic guinea-pigs varies within wide limits and no difference can be established in this respect between the two groups.

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LXVIII. A NOTE ON THE REPRECIPITATION OF THE ANTISCORBUTIC FACTOR FROM DECITRATED LEMON JUICE.

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In a recent paper [Zilva, 1930] it was pointed out that when the active precipitate obtained by precipitation with lead acetate at p_H 7·2 from decitrated lemon juice, previously cleared with neutral lead acetate, was dissolved in acetic acid and the reaction brought up with ammonia to p_H 7, no precipitate was formed at that hydrogen ion concentration. Only when the p_H was raised to 8 a precipitate began to appear and the maximum precipitation was obtained between p_H 8 and 9 only. This fraction was found to be totally inactive in doses equivalent to 5 cc. of the original juice. The observation was not in accord with that made by Grettie and King [1929] and Sipple and King [1930] (the latter cleared their decitrated juice in the first place with alcohol) who obtained a second precipitate at p_H 7·2–7·4 which, they claimed, showed very little loss in activity after the reprecipitation.

In dissolving the first precipitate in the above experiment an excess of 10 % acetic acid, namely a volume equal to that of the original juice from which the precipitate was obtained, was used and this yielded a very clear solution. It has been since found that if less acetic acid is used the p_H at which the second precipitate begins to appear is considerably lowered. Thus, with a certain juice it was possible, by using the least quantity of 10 % acetic acid necessary to dissolve the precipitate (about 10 cc. per 50 cc. of the original juice), to lower the p_H at which the precipitate first appears to 6·2–6·9 (average 6·4) instead of 8 previously observed, and consequently a considerable precipitate was obtained when the p_H was further raised to 7·4. The first precipitate began to appear at p_H 5·5–6·0 (average 5·7). On testing the second precipitate for its antiscorbutic potency, it was found that an equivalent of 5 cc. of the original decitrated juice gave very nearly the same degree of protection as an equivalent of 1·5 cc. of the first precipitate; in other words, a loss of rather less than 66 % was incurred in the reprecipitation. In estimating the dry matter in 7 samples of the first and second precipitates, decomposed with $MgSO_4$, during the progress of the biological test, it was found that the loss of solids varied from day to day but that the average amount

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of inactive material removed by the second precipitation amounted to only 33 %, a lower figure than that of the loss incurred in antiscorbutic activity. No purification was therefore effected in this case.

It must be further pointed out that the reducing capacity for phenolindophenol of the first precipitate of this batch averaged 11 cc. of 0·02 % phenolindophenol per equivalent of 5 cc. of the original juice, the highest figure so far obtained by the author. In the second precipitate there was a loss of 40–50 % of this reducing capacity. With the average preparation, however, such a loss would have brought the reduction down to a level which experience has shown to be characteristic of inactive fractions, and a risk is thus run of totally inactivating the fraction by a second precipitation, even when it is brought down at about p_H 6·4. This point will be dealt with in detail in a subsequent publication.

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LXIX. THE EFFECT OF DESICCATION UPON THE NUTRITIVE PROPERTIES OF EGG-WHITE. II.

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In a previous paper [Boas, 1927] it was shown that dried egg-white, when used as the sole source of protein in a diet for young rats, produces a train of characteristic symptoms—notably dermatitis, baldness and spastic gait—terminating in death in from 4 to 6 weeks. It was shown further that this syndrome can be completely prevented if certain substances such as yeast, potato, arrowroot, commercial caseinogen, fresh egg-white and others are added to the diet, and the presence of an unknown protective dietary factor X in these substances was postulated. The symptoms were produced only when the egg-white was dried in the undenatured state, but the results were the same whether dehydration was carried out by evaporation in air at 37°, *in vacuo* in the presence of H_2SO_4 at room temperature, or by treatment with alcohol. The two following alternative hypotheses were advanced as possible explanations of the phenomena:

(*a*) that dried egg-white is lacking in some essential dietary factor, which is supplied by protective factor X;

(*b*) that a toxic substance is formed in dried egg-white as a result of desiccation, and that this is neutralised in some way by protective factor X.

The investigation has since been carried a stage further and as there is at present no prospect of continuing it, it seems best to publish the results so far obtained although the work is still incomplete. There are now other workers in the field and it is hoped that the experiments described in this paper may be of assistance to them.

Findlay and Stern [1929], using the same diet as the author, obtained similar results and confirmed the protective power of raw potato, yeast, raw egg-white, egg-yolk and cow's milk. They found raw liver to have a high potency. They suggest that the condition produced in the rats fed upon the dried egg-white diet is analogous to "pink disease" (Swift's disease) in children, the clinical picture of nervous and cutaneous symptoms being similar. An extensive histological study of the rats showed that the most important changes were in the skin, where there was active keratinisation,

and in the spinal cord, where a diffuse infiltration of small round cells into both grey and white matter was revealed. A mild case of Swift's disease examined by them showed a similar infiltration into the spinal cord. Since Findlay and Stern were able to produce the characteristic symptoms in newborn and suckling rats by feeding the mother during pregnancy and lactation on the dried egg-white diet, they favour the hypothesis that the dried egg-white is deficient in some dietary essential, but they admit that the theory that a toxic substance is involved is not excluded by this evidence.

Parsons [1931] has also produced the dried egg-white syndrome in rats, which for the most part received diets containing as much as 66 % of protein in the form of dried egg-white, raw or cooked. In her description of the symptoms Parsons mentions in addition a scaly condition of the tails which the present author has not observed. Parsons found that dried beef-liver, if constituting 20 % of the diet, protected the rats but that 10 % did not and that 20 % of dried yeast was also ineffective.

The present paper is mainly concerned with a series of tests, some of a curative and some of a preventive nature, in which the effects of using some of the different constituents of egg-white, fresh or dried, as the source of protein were investigated. Such tests were expected to produce evidence which was in favour of or could be set against one or other of the alternative hypotheses described above.

Description of diets and materials used.

Table I gives full details of the constitution of the various diets used in these experiments. Each rat received in addition to the diet 3 to 5 drops of cod-liver oil daily. All the egg-white diets were dissolved in water and coagulated by heat before incorporation in the diet.

Table I. *Details of the diets used.*

	DW diet	DG diet	Diets containing separate egg-white proteins (1 *a*)	(1 *b*)	2	3	4	5	6	7	Casein-ogen diet	Liver diet	Steak diet
Dried egg-white	100	100	—	—	—	—	—	—	—	50	50	50	50
Pure ovalbumin solution	—	—	100 (dry)	—	—	—	—	—	—	—	—	—	—
Dried pure ovalbumin	—	—	—	100	—	—	—	—	—	—	—	—	—
Dried ovoglobulin*	—	—	—	—	200	—	—	—	16	—	—	—	—
Dried egg-white less globulins	—	—	—	—	—	100	—	—	—	—	—	—	—
Dried albumins and ovomucoid	—	—	—	—	—	—	100	—	91	50	—	—	—
Albumins and ovomucoid from dried egg-white	—	—	—	—	—	—	—	100	—	—	—	—	—
Fresh raw ox-liver	—	—	—	—	—	—	—	—	—	—	—	250	—
Fresh raw beef steak	—	—	—	—	—	—	—	—	—	—	—	—	250
Purified caseinogen	—	—	—	—	—	—	—	—	—	—	50	—	—
Wheat starch	250	—	250	250	250	250	250 or 250	250 or 250	250	250	250	250	250
Glucose	—	250	—	—	—	—			—	—	—	—	—
Hardened cotton seed oil	75	75	75	75	75	75	75	75	75	75	75	75	75
Marmite	25	25	25	25	25	25	25	25	25	25	25	25	25
Salt mixtures†	25	25	25	25	25	25	25	25	25	25	25	25	25
Distilled water	300	300	—	300	300	300	300	300	300	300	300	150	150

* This was 50 % NaCl.

† McCollum, Simmonds and Pitz [1917].

The materials used were prepared as follows.

Dried egg-white. See Boas [1927].

Egg-white fractions. (1 *a*) *Pure ovalbumin.* Ovalbumin crystals were prepared from fresh egg-white by the method of Hopkins and Pinkus [1898], dissolved in water and dialysed against tap-water until free from sulphate, a little toluene being added to prevent decomposition.

(1 *b*) *Pure ovalbumin, dried.* Fraction (1 *a*) was dried at 37°.

(2) *Ovoglobulin, dried.* To raw fresh egg-white an equal quantity of a saturated solution of ammonium sulphate was added and the precipitate filtered off. This was treated with a dilute solution of sodium chloride in order to dissolve the ovoglobulin. Most of the ovomucin remains undissolved [Hektoen and Cole, 1928] and can be filtered off. The ovoglobulin was then treated again with a saturated solution of ammonium sulphate and the precipitate further purified by redissolving it in water and precipitating a third time. It was then dissolved, dialysed as above, taken up with a dilute solution of sodium chloride and dried as usual. This preparation contains a little ovalbumin as well as the ovoglobulin.

(3) *Filtrate after removal of the globulins, dried.* Method (*a*). Raw fresh egg-white from which the globulins had been removed by half-saturation with ammonium sulphate was dialysed as above and dried in the usual way.

Method (*b*). To raw fresh egg-white four volumes of water were added, the whole well mixed and made neutral to litmus with dilute acetic acid. The precipitate of globulins was filtered off and the filtrate dried.

(4) *Albumin and ovomucoid fraction from fresh egg-white, dried.* Fresh raw egg-white, from which the globulins had been removed by half-saturation with ammonium sulphate, was treated with excess of solid ammonium sulphate until no further precipitate could be produced and the filtrate was protein-free. The precipitate was filtered off, dissolved in water, reprecipitated and again filtered off. After dissolving in water it was dialysed and dried.

(5) *Albumin and ovomucoid fraction from dried egg-white, dried.* 200 g. of dried egg-white were dissolved in 1600 cc. of water. From this solution the albumin and ovomucoid fraction was prepared as under (4).

Purified caseinogen. This was prepared by the method described by Chick and Roscoe [1928], to whom I am indebted for the sample used here.

Yeast extract. Pressed washed yeast was heated for 5 hours at 120° and then stirred into a large volume of boiling dilute acetic acid (0·01 %) and boiled for 5 minutes. It was then filtered and the filtrate concentrated so that 1 cc. was equivalent to 0·7 g. of the original dry yeast.

Yeast residue. The residue from the yeast extract was subjected to two further similar extractions and then dried at 37°. 1 g. of the residue was equivalent to 2·2 g. of the original dry yeast.

Experiments on the purified proteins of egg-white.

Crystalline ovalbumin.

Assuming that crude raw egg-white contains some essential accessory substance X which is destroyed during drying (hypothesis (*a*) above) one might expect that a pure crystalline constituent protein such as ovalbumin would be freed from this factor. The use of pure ovalbumin as the sole protein of the diet would in that case be followed by the development of the same symptoms as are produced by the use of dried egg-white. Crystalline ovalbumin, prepared as described above, was used as the sole source of protein in the diet of two rats (see Table II), which had developed the usual symptoms as a result of receiving the dried egg-white diet (DW) for 8 weeks. Their condition immediately improved and rapid growth was resumed. It was, however, possible that the essential factor might still remain adherent to the albumin even after crystallisation. The solution of pure ovalbumin was therefore dried at 37°. Curative experiments on 3 rats (see Table II) proved it to have suffered no change in nutritive property.

Table II. *Results obtained when rats which had developed the symptoms as a result of receiving the dried egg-white (DW) diet were given instead diets containing different egg-white fractions as the source of protein.*

Litter No.	Rat No.	Length of time on DW previously (days)	Condition of rat when diet changed: Symptoms	Condition of rat when diet changed: Weight	New diet No.*	Result of change of diet: Symptoms	Result of change of diet: Weight g.
78	657 ♀	58	Severe	Falling	(1 *a*)	Healing	Gain of 27 in 16 days
78	658 ♂	58	,,	,,	(1 *a*)	,,	,, 24 ,, 16 ,,
81	686 ♂	Steak diet 51 DW 8	Marked	,,	(1 *b*)	,,	,, 32 ,, 11 ,,
82	697 ♂	21	Appearing	Rising	(1 *b*)	,,	,, 67 ,, 18 ,,
82	699 ♀	21	,,	,,	No change	Worse	,, 9 ,, 18 ,,
84	715 ♂	33	Severe	Falling	(1 *b*)	Healing	,, 37 ,, 18 ,,
84	716 ♂	42	,,	,,	4	,,	,, 18 ,, 14 ,,
84	717 ♂	42	,,	,,	4	,,	,, 22 ,, 18 ,,
84	718 ♀	42	Marked	,,	4	,,	,, 29 ,, 18 ,,
84	714 ♂	42	Severe	Stationary	No change	Worse	Loss of 6 ,, 9 ,,
84	719 ♂	42	,,	,,	,,	,,	,, 4 ,, 12 ,,
84	720 ♀	42	,,	,,	,,	,,	,, 7 ,, 12 ,,
86	731 ♀	36	Marked	,,	6	Healing	Gain of 26 ,, 12 ,,
86	735 ♂	36	Severe	,,	6	,,	,, 12 ,, 12 ,,
86	732 ♀	36	Marked	Falling	6	,,	,, 31 ,, 12 ,,
86	734 ♂	36	Severe	,,	6	,,	,, 28 ,, 12 ,,
86	736 ♂	36	Marked	,,	No change	Worse	Loss of 8 ,, 12 ,,
86	737 ♂	36	Severe	Stationary	3	Died in 7 days	
86	739 ♂	36	,,	Falling	3	Worse	Loss of 5 in 14 days
86	738 ♂	36	,,	Stationary	4	Healing	Gain of 29 ,, 16 ,,
89	761 ♀	35	Marked	,,	2	,,	,, 48 ,, 21 ,,

* See Table I.

These results are opposed to the theory that the symptoms caused by the ingestion of dried egg-white as the sole protein are caused by a deficiency of some dietary essential. One is, therefore, forced to conclude that when egg-white is dried some toxic substance is formed (hypothesis (*b*)).

The source of the toxic substance.

In the hope of determining from which of the constituents of egg-white the toxic substance is derived some of the other proteins contained in egg-white were examined separately. According to Hektoen and Cole [1928] there are five different proteins in egg-white, ovoglobulin and ovomucin together constituting the fraction precipitable by half-saturation with ammonium sulphate, ovalbumin and conalbumin both precipitated by saturation with ammonium sulphate, the first crystallisable and the second non-crystallisable and ovomucoid. The albumins constitute about 80 % of the total protein, the globulins 7 % and the ovomucoid 10 %.

Use was made of this classification of Hektoen and Cole in preparing the egg-white fractions described above. From these egg-white diets (2), (3), (4) and (6) in Table I were made up and given to rats which had developed the usual symptoms as the result of receiving the DW diet for from 21 to 42 days.

The results of the change of protein are shown in Table II. It will be seen that diets (2), (4) and (6) were harmless, their replacement of the DW diet being followed by healing and resumption of growth. Diet (3), however, containing the whole egg-white dried after removal of the globulin fraction, appeared to be toxic. To confirm this result, 3 rats of one litter were fed from weaning on diet (3), 5 rats from the same litter receiving the DW or DG diets. The remaining 3 rats in the litter received diet (5), containing as the sole protein the albumins and ovomucoid fraction prepared from dried egg-white. At the end of 6 weeks it was found that all the rats with the exception of those fed on diet (5) had developed the usual symptoms, thus confirming the toxic nature of egg-white dried after removal of the globulin fraction. This experiment demonstrated also the important fact that although dried egg-white is toxic, it is possible to prepare from it a non-toxic fraction. This provides further convincing proof that dried egg-white is not lacking in some unknown essential. The growth curves of the rats in this experiment are shown in Fig. 1.

These results show that neither ovoglobulin, conalbumin, ovalbumin nor ovomucoid produces the toxic substance when it is dried. Since the toxic substance is produced when the albumins, the ovomucoid and the protein-free mother-liquor are dried together, ovomucin can also be exculpated. It follows, therefore, that the toxic substance is derived, either from some constituent of the mother-liquor or alternatively from one of the albumins or the ovomucoid when these are dried in the presence of the mother-liquor. Against the latter hypothesis must be set the harmless nature of the albumins and ovomucoid fraction when separated from the rest of the toxic dried egg-white.

One is forced to conclude, therefore, that during desiccation of egg-white a toxic substance is formed from some non-protein constituent and that this substance remains present in the mother-liquor after removal of the proteins by precipitation with ammonium sulphate. This hypothesis cannot be regarded as fully established until it has been shown that the addition of the

dried mother-liquor to an otherwise harmless diet results in the production of the characteristic syndrome, an experiment which has not as yet been carried out.

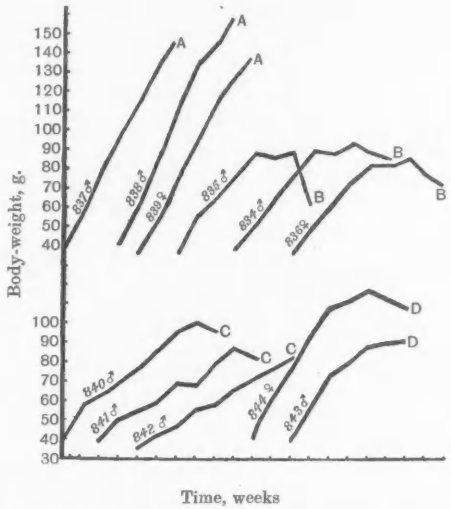

Fig. 1. Growth curves of rats fed from weaning on diets containing as the source of protein:
A. Albumin and ovomucoid fraction from dried egg-white.
B. Egg-white dried after removal of the globulin fraction.
C. Dried egg-white.
In A, B and C the carbohydrate was glucose, in D wheat starch.
All rats were from the same litter.

The protective factor X.

Solubility. It was shown previously [Boas, 1927] that the protective factor X appeared to be insoluble in water or alcohol. Further evidence on this point has been obtained.

(1) The factor is not removed from caseinogen by repeated washing with dilute acetic acid followed by extraction with hot alcohol. Rats reared from weaning on the caseinogen diet described in Table I, in which the protein consisted of dried egg-white and purified caseinogen in equal quantities, grew well and appeared in perfect health. (It was shown before [Boas, 1927] that a reduction of the dried egg-white in the diet to one-half the usual amount without the addition of any other substance did not decrease the severity of the symptoms produced.)

(2) Only a small amount of the factor is extracted from fresh washed yeast by boiling in dilute acetic acid (0·01 %). A daily dose of yeast extract equivalent to 1·4 g. of dried yeast did little to prevent the syndrome in rats fed upon the DW diet, whereas a dose of the yeast residue equal to 1·3 g. of dried yeast gave good protection.

(3) Fresh egg-white possesses protective power. Complete protection resulted when fresh egg-white was ingested in a proportion to dried egg-white of one to one, but when the ratio was one to three, only partial protection was afforded. It has now been found that when a precipitate of albumins and ovomucoid is formed by saturation with ammonium sulphate of fresh egg-white, from which the globulins have been removed, the protective substances are also carried down. This fraction can neutralise the toxic material in dried egg-white if it is given to rats in the proportion of one part of the fraction to one part of dried egg-white.

The experiments briefly described above are shown in full detail in Table III.

Table III. *Details of experiments showing the presence of protective factor X in certain substances.*

Litter No.	Rat No.	Diet*	Length of exp. (days)	Gain in body-weight g.	Condition of rat at close of exp.
83	705 ♀	DW	41	63	Marked symptoms
83	712 ♂		41	79	,,
83	707 ♀	Caseinogen	41	67	Good condition
83	708 ♀		41	79	,,
83	709 ♂		41	111	,,
83	710 ♂		41	97	,,
82	695 ♂	DW	35	—	Dead
82	699 ♀		42	48	Marked symptoms
82	700 ♀		42	42	,,
82	702 ♀	DW + daily dose of 2·0 cc. yeast extract (last 3 weeks)	42	66	Moderate symptoms
82	703 ♀		42	69	,,
82	696 ♂		42	42	,,
82	694 ♂	DW + daily dose of 0·6 g. yeast residue (last 3 weeks)	42	85	Good condition
82	704 ♀		42	72	,,
90	772 ♀	DW	47	44	Moderate symptoms
90	773 ♀	Egg-white fraction, diet 7	47	88	Good condition
90	771 ♂		47	85	,,
81	683 ♂	DW	42	59	Severe symptoms
81	684 ♂		42	27	,,
81	690 ♀		42	25	Marked symptoms
81	685 ♂	Steak diet	42	113	,,
81	686 ♂		42	91	,,
81	687 ♂		42	89	,,
81	691 ♀		42	77	,,
81	688 ♂	Liver diet	23	107	Excellent condition
81	689 ♂		23	64	,,
81	692 ♀		23	62	,,
81	693 ♀		23	71	,,

* See Table I.

Distribution in nature. The paucity of the protective factor X in lean meat has been confirmed. Rats reared on a diet (see Table I) in which the protein consisted of 50 g. of dried egg-white and 250 g. of raw lean beef steak (68 g. dry weight) showed a condition little superior to that of the controls receiving DW diet. On the other hand, raw liver is a rich source, for 250 g. (83 g. dry weight) of raw ox-liver proved amply sufficient to counteract the effects of 50 g. of dried egg-white. Parsons [1931] found that the amount of dried beef-

liver needed to neutralise 66 g. of dried egg-white was between 10 and 20 g. and that 20 g. of dried yeast were inadequate. On the other hand, I have found that a rat ingesting from 1 to 2 g. of dried egg-white daily was kept in good health by a daily dose of 0·4 g. of dried yeast. Findlay and Stern [1929] have also confirmed the high protective potency of dried yeast.

Refection.

Some interesting light has been thrown on the problem by the discovery that rats showing characteristic symptoms when fed upon dried egg-white sometimes exhibit spontaneous cures. At the same time they produce bulky faeces containing undigested starch. These effects appear to be of a similar nature to the spontaneous cures occasionally observed in the case of rats reared upon a diet devoid of water-soluble vitamins [Fridericia *et al.* 1927; Roscoe, 1927]. In this condition, called refection by Fridericia, the rats pass bulky faeces white in colour with a large content of undigested starch and as long as such faeces are produced the rat is able to grow and enjoy perfect health in spite of the absence of water-soluble B vitamins from the diet. Refection can be transmitted by feeding the faeces of a refected rat and Fridericia *et al.* have postulated the existence of a virus of refection, cultivated in the intestine of the affected rat and passing out in the faeces.

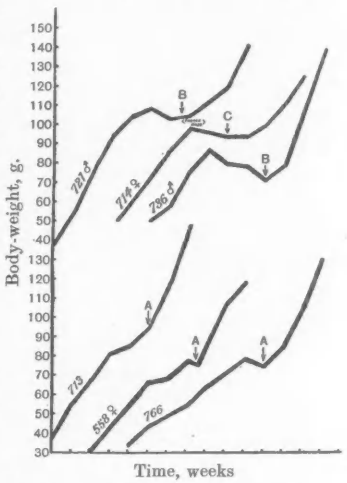

Fig. 2. Growth curves of rats showing:
At A. Spontaneous cures.
At B. Cures induced by a daily dose for 7 days of 0·7 g. of the faeces of a refected rat.
At C. Cure induced by a daily dose for 12 days of 0·5 g. of the faeces of rat 713, showing a spontaneous cure.
All rats were receiving the DW diet throughout.

In the spontaneous cures observed in rats receiving the dried egg-white diet the condition could also be passed from one rat to another by ingestion of the faeces. Furthermore, rats showing characteristic symptoms on the dried egg-white diet exhibited cures after receiving doses of the faeces from refected rats (see Fig. 2). It appears, therefore, that the agency in both cases of refection may be identical and this suggests two possibilities. If, as Fridericia suggests, refection is due to the growth in the gut of an organism which synthesises the members of the vitamin B complex, then this organism may also be supposed to synthesise the protective factor X. This is not improbable for yeast has been found to be a rich source of all these factors. On the other hand, the spontaneous cures of the rats fed on the dried egg-white diet may be simply due to some union of the toxic substance with the undigested starch thus preventing absorption of the former.

Discussion.

The weight of the evidence offered above is certainly on the side of the hypothesis that dried egg-white contains some toxic substance created during the process of drying. It is for the most part opposed to the theory that the nutritive disorder is due to a deficiency. These facts afford little support to Findlay and Stern's proposed identification of the egg-white syndrome with Swift's disease. Their case is based on pathological and clinical findings and until evidence of a therapeutic or dietetic nature is obtained must be regarded as unproved.

The protein-free mother-liquor has been suggested as the source of the toxic substance, but this deduction is based on a process of exclusion and much further work is needed before it can be regarded as established.

It has already been pointed out [Boas, 1927] that the protective factor X presents in its distribution in nature striking analogies with the water-soluble B vitamins, in particular with vitamin B_2. Its presence, however, in purified caseinogen which, so far as is at present known, contains none of the members of the vitamin B complex, and its absence from marmite clearly prove that it is not identical with any of these factors. It seems highly probable that it will ultimately prove to be a closely allied substance.

The conception of a toxic substance in the diet, neutralised by a specific accessory factor, is not a new one in dietetics. Mellanby [1926] has demonstrated a toxamin in oatmeal the action of which is antagonised by vitamin D, and there is considerable evidence that the harmful effects of an excess of certain proteins in the diet can be counteracted by a factor probably identical with one of the constituents of the water-soluble B complex [Hartwell, 1922, 1924, 1925; Reader and Drummond, 1926; Hassan and Drummond, 1927; Parsons, 1931].

Summary.

1. Pure ovalbumin, prepared by crystallisation from crude egg-white, forms a satisfactory protein for young rats. The same is true of ovoglobulin and of the total albumin and ovomucoid fraction.

2. Desiccation does not alter the nutritive properties of these materials as is the case with crude egg-white.

3. It therefore appears probable that the alteration in nutritive value observed when egg-white is dried is due not to the loss of some essential factor but to the formation of a toxic substance.

4. Evidence is adduced suggesting that the toxic substance is formed from some non-protein constituent of the egg-white.

5. It is possible to prepare from dried egg-white a protein fraction uncontaminated by this toxic substance.

6. The protective factor X which is capable of neutralising the toxic substance in dried egg-white is present in raw liver, in a dried preparation of

the total albumin and ovomucoid fraction of egg-white, but not in raw beef steak.

7. It is only partially extracted from yeast by boiling dilute acetic acid.

8. It is not removed from caseinogen by washing with dilute acetic acid and extraction with alcohol.

9. Rats suffering from the effects of the dried egg-white can become spontaneously refected in a similar manner to that observed by Fridericia and his colleagues and by Roscoe in the case of rats fed upon diets devoid of water-soluble B vitamins.

10. This condition can be transmitted from one rat to another by ingestion of the faeces.

11. Refection can be induced in rats, receiving the dried egg-white diet, by feeding the bulky white faeces of refected rats on diets deprived of B vitamins. This suggests that the agency is the same in both cases.

I should like to record my thanks to Sir Charles Martin and to Dr H. Chick for their advice and help throughout the course of the investigation.

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LXX. THE NATURE OF THE LIPOID MATTER EXTRACTED FROM GREEN LEAVES (SPINACH AND CABBAGE).

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The unsaponifiable fraction from the spinach leaf.

Clenshaw and Smedley-MacLean [1929] examined the unsaponifiable matter of the lipoid matter of spinach leaves and isolated from it (1) carotene, (2) a saturated hydrocarbon melting at 68–68·5° which they identified as hentriacontane $C_{31}H_{64}$, and (3) a sterol which forms less than 5 % of the total unsaponifiable matter. The same authors also examined the leaves of green cabbage and showed that they contained a hydrocarbon, which they did not completely characterise, similar to that present in spinach. This was accompanied by a substance containing oxygen which did not appear to be represented in spinach and which was reserved for further investigation.

Shortly afterwards Heyl, Wise and Speer [1929] published the results of an examination of spinach fat in which very much larger quantities of material had been worked up than had been used by the previous observers. The conclusions of these authors differed so widely from those of Clenshaw and Smedley-MacLean that it seemed desirable to repeat the investigation of spinach fat and to endeavour to reconcile the discrepancies.

The hydrocarbon isolated by Heyl, Wise and Speer melted at 67·5–68·5° and contained C, 84·8 %, H, 15 %: to this they ascribed the formula $C_{20}H_{42}$, apparently on the ground of a single molecular weight determination by Rast's method. Since the analyses and melting-points of the hydrocarbons isolated in the two investigations were completely in agreement and corresponded with those of the hydrocarbon $C_{31}H_{64}$, it appeared necessary to carry out a number of determinations of the molecular weight of the hydrocarbon by Rast's method [1922] to see how far they agreed and also to confirm the composition of the hydrocarbon by submitting it to X-ray examination.

In our hands the values obtained for the molecular weight of the hydrocarbon were 409, 432 and 424, while another observer found 405 and 420 for the same material. We can offer no explanation of the value of 285, the single observation recorded by Heyl, Wise and Speer. Our experience indicates that while Rast's method is exceedingly useful in fixing the approximate molecular weight of a hydrocarbon, it does not afford a basis for a decision between

adjacent members of a homologous series when carried out under the ordinary laboratory conditions of determining melting-points.

We are indebted to Dr Piper for carrying out the X-ray examination. He reports that the hydrocarbon gave a very good photograph spacing 41·3 Å units to ½ %. It gave a large number of orders—about ten measurable—and was probably a highly pure specimen of the hydrocarbon containing 31 carbon atoms.

There is no doubt therefore that Clenshaw and Smedley-MacLean were correct in assigning the formula $C_{31}H_{64}$ to the hydrocarbon present in the unsaponifiable matter of spinach. From the same material Heyl, Wise and Speer claim to have isolated two other substances; (1) an alcohol melting at 76–77° to which they ascribed the formula $C_{22}H_{46}O$ as the result of analysis and of a single recorded observation by Rast's method, and (2) a substance melting at 87–88° containing C, 77·7 %, H, 13·7 %, for the molecular weight of which a single determination by Rast's method gave 272. Presumably as the result of an error in calculation the formula $C_{24}H_{50}O_2$ corresponding with a molecular weight of 370 is ascribed to this substance. Having worked up considerably more material than in the earlier investigation of Clenshaw and Smedley-MacLean, we succeeded in isolating a substance containing oxygen, which agreed in melting-point and in composition with the alcohol described by Heyl, Wise and Speer. The complete separation of all traces of hydrocarbon from the spinach alcohol is attended with some difficulty. We found that the alcohol formed an acetate melting at 61·5° and a urethane melting at 64–65° and closely resembled *n*-ceryl alcohol. A specimen of the latter was prepared from Chinese wax and the addition of this alcohol, its acetate and urethane respectively to the similar compounds derived from the spinach alcohol caused no depression in melting-point. The alcohol described by Heyl, Wise and Speer as $C_{22}H_{46}O$ is therefore identical with ceryl alcohol.

Alcohol	C %	H %	Mean mol. wt. (Rast)	M.P.
Ceryl alcohol ($C_{26}H_{54}O$)	81·67	14·13	382	78–79°
Alcohol from Spinach:				
(*a*) Heyl, Wise and Speer	80·9	14·2	272	76–77°
(*b*) Collison and Smedley-MacLean	81·32	13·74	353 365 421	77–78°

In our investigation the total amount of dry spinach leaves worked up was 6·1 kg. whereas the American investigators started with 68 kg. This probably explains why we did not succeed in obtaining any of the oxygen-containing substance melting at 87–88°, 0·2 g. of which was isolated by Heyl, Wise and Speer.

The sterol from spinach was of particular interest. Unfortunately the amount isolated was not sufficient for an extended investigation. Our analyses agreed with those of the above observers and we confirm the formula $C_{27}H_{46}O$. It is dextro-rotatory, melts at 164°, gives a Salkowski reaction with the reddish

colour in the sulphuric layer, and is highly unsaturated giving an iodine value by Hubl's method of 245. It closely resembles in its properties the much lower-melting zymosterol with which it is isomeric.

The fatty acids of the spinach leaf.

These were examined and palmitic and cerotic acids identified, confirming the result of Speer, Wise and Hart [1929]. From 3600 g. dried spinach leaves, 69·12 g. of material soluble in light petroleum were extracted. On saponifying this with a cold alcoholic solution (6 %) of sodium ethoxide, 25·67 g. of unsaponifiable matter were obtained and after acidification and further purification by boiling with charcoal in solution in light petroleum, 10·5 g. of fatty acids were isolated. The acid fraction was separated by means of the solubilities of its lead salts in ether into a liquid and a solid fraction. The latter was separated into two parts which showed the following properties:

	M.P.	Mol. weight (by titration)
Fraction I	74·5–75·5°	406
(Several times recrystallised)	78–78·5°	—
Cerotic acid	78–79°	410
Mixed M.P.	78–79°	—
Fraction II	50°	266
(Several times recrystallised)	59–61°	—
Palmitic acid	62°	256

The molecular weight of the liquid portion of acids was found by titration to be 259 and its iodine value 205·8; on bromination of this portion, oleic and linolenic acids were identified. A doubly unsaturated acid was also present, but the solubility of its tetrabromide in light petroleum was greater than that expected for the bromide of linoleic acid. It is possible therefore that the doubly unsaturated acid present is either an isomeride of linoleic acid or else a lower homologue. The quantity of material at our disposal did not permit us to investigate this further.

The unsaponifiable fraction from the cabbage leaf.

The amount of lipoid material extracted by light petroleum from green cabbage leaves which had previously been dried in air at 37° was similar to that which we had obtained from spinach leaves, namely about 2 %, of the weight of dried leaves. Approximately one half of this consists of unsaponifiable matter. Chibnall and Channon [1927, 1] carried out an investigation on the lipoid matter of cabbage, prepared in a somewhat different manner. Their method was to mince the cabbage leaves, to add an equal volume of water to the minced leaves and to express the juice, which was then coagulated at 70°. The coagulum was separated by filtration and pressed so that the residue contained about 40 % of water. The residue when treated with ether gave an extract which was dark green in colour and contained chlorophyll and its decomposition products, whereas the extract which we obtained by the

direct treatment of the leaves (dried at 37°) with light petroleum was light yellow and almost free from chlorophyll.

The lipoid matter from the petroleum extract was saponified in the cold by means of 6 % alcoholic sodium ethoxide and gave about half its own weight of unsaponifiable matter. The iodine value of the unsaponifiable matter obtained from the green leaves of large summer cabbage was 100 compared with a value of 57·5 for that obtained from the pale yellow heart leaves of the same cabbage. The iodine value of the unsaponifiable matter prepared from a green unheaded winter cabbage was 58·5. The difference in iodine value is probably explained by differences in carotene content [Collison, Hume, Smedley-MacLean and Smith, 1929] for the carotene content of white leaves is much less than that of green leaves and the winter cabbage also proved less effective as starting material for the preparation of carotene than the large-headed summer cabbage. The iodine value of carotene determined by the same method as that used for the unsaponifiable matter of cabbage was 330 and the proportion of carotene present in the unsaponifiable matter must therefore greatly influence the iodine value of the unsaponifiable material.

The unsaponifiable material was treated with hot alcohol: most of the carotene remained in the sticky insoluble residue and from this a specimen melting at 178° was prepared. In the hot alcoholic solution there remained the hydrocarbon and oxygen-containing compound previously noticed by Clenshaw and Smedley-MacLean. A full description of these substances was published by Channon and Chibnall [1929] shortly afterwards and they were identified as *n*-nonacosane, $C_{29}H_{60}$, and *n*-dimyristyl ketone respectively. We found that the best method of separating these substances is by washing the mixture with small quantities of cold light petroleum, in which the hydrocarbon is more readily soluble. When the mixture containing a large proportion of the ketone was converted to the oxime by the method described by Channon and Chibnall and the substance obtained after oximation dissolved in acetic acid, the precipitate which separated when the solution was cooled melted at 55°–60°. On the addition of alcohol to the filtrate, short needles separated melting at 57·5°. When these were mixed with palmitone oxime (M.P. 58–59°) the melting-point of the mixture was depressed by 5°. The oxime of the dimyristyl ketone described by Channon and Chibnall melted however at 50–51°. In another similar preparation of the oxime starting with a different batch of material, the oxime which separated melted at 52–53°. Both specimens, melting respectively at 52–53° and at 57·5°, gave the same results on analysis and from both on treatment with acid the same ketone melting at 80° was recovered. We are now investigating the reason for this difference in melting-point, which we cannot at present explain.

The fatty acids of the cabbage leaf.

From 2800 g. of dry cabbage leaves, 14·3 g. of fatty acids insoluble in water were separated and these were divided by the usual lead salt method into 7·21 g. of liquid and 1·6 g. of solid acids.

The solid acid fraction was separated by repeated crystallisation into two parts melting respectively at 62–64° and 80–80·5°. So little material was available that the definite identification of these fractions presented considerable difficulties. The lower melting fraction appeared to consist chiefly of palmitic acid. The molecular weight determined by titration of the fraction of M.P. 80° was 357, a value intermediate between those required for behenic acid ($C_{22}H_{44}O_2$, M.P. variously given as 77–78°, 80–82°, 84°) and lignoceric acid ($C_{24}H_{48}O_2$, M.P. 80·5°). An ethyl ester was prepared from the small amount of acid available and melted at 51–52°. The ethyl ester of behenic acid melts at 56–57°.

Chibnall and Channon [1927, 2] give the following figures for the identification of the fractions from the 1·45 g. of solid acids isolated by them from cabbage:

		C %	H %	M.W.	M.P.
Fraction (1)	0·38	75·23	12·60	271	54–55°
(2)	0·65	75·18	12·43	262	54–55°
(3)	0·34			266	—

They point out that these figures would be given by a mixture of 30 % of stearic and 70 % of palmitic acid and hence they assume that these acids are present. A mixture of 90 % palmitic acid with 10 % behenic acids would give the following figures:

C %	H %	M.W.
75·19	12·61	264

From the evidence now brought forward it is clear that the evidence for the presence of stearic acid in the cabbage leaf is very doubtful, since in this plant the palmitic acid present is associated with a small proportion of a higher saturated acid, probably behenic.

In view of the recent identification of the liquid acids as oleic, linoleic or its homologue, and linolenic, we did not further investigate the liquid fraction of the acids.

General remarks on the lipoid material extracted from green leaves.

From the investigation of the leaves of cabbage and spinach and from the evidence available, chiefly in the work of Power, Tutin and their collaborators, it seems probable that the higher members of the normal paraffin series and their derivatives must play an important part in the metabolism of the plant leaf.

Speaking generally the bulk of the acids of the leaf seem to be the unsaturated acids. Linolenic, linoleic or an isomeride, oleic and an unsaturated acid containing 16 carbon atoms have been recorded. The proportion of solid acids is small and of these palmitic acid seems to be an almost invariable constituent and in a large proportion of instances has been isolated in the pure state. Smaller amounts of one or other of the higher saturated fatty acids appear commonly to be present. Stearic acid is frequently noted though the evidence for its presence often consists of the analysis of a fraction of fatty acids of lower melting-point and slightly higher carbon content than palmitic acid. This mixture is sometimes obtained by recrystallisation and sometimes by saponification of a fraction obtained by distillation of the methyl esters, but in many cases the evidence adduced for the presence of stearic acid is unconvincing. From the evidence in the literature cerotic appears to occur most commonly of the higher fatty acids and several instances are recorded of the occurrence of arachidic, behenic, and melissic acids.

The higher fatty alcohols also occur; ceryl alcohol has been found in spinach leaves in the present investigation, in hops [Power, Tutin and Rogerson, 1913], in *Prunus serotina* [Power and Moore, 1909] and in other plants.

The occurrence of these higher fatty alcohols and acids is of interest in connection with the presence of the higher paraffin hydrocarbons in leaves. Thorpe and Holmes [1901] isolated from tobacco leaves two hydrocarbons melting respectively at 59·3–59·8° and 67·8–68·5°, and identified them as the normal paraffin hydrocarbons containing 27 and 31 carbon atoms. Since that time, the analyses of a large number of leaves, stems and flowers have been made chiefly by Power, Tutin and their collaborators. In a somewhat cursory survey of the literature we have noted the isolation of these hydrocarbons from 39 different plants. From 21 of these hentriacontane ($C_{31}H_{64}$), melting at 68° or in its near neighbourhood, has been separated. This hydrocarbon is the one most often recorded and is presumably derived from palmitic acid, by conversion of the latter to palmitone and by the subsequent reduction of the latter. We can however in the papers we have examined find no record of the presence of palmitone in the same material and we have been unable to find any evidence of the presence of this ketone in the spinach leaf. The presence of pentatriacontane $C_{35}H_{72}$ which bears a similar relation to stearic acid to that existing between hentriacontane and palmitic acid is recorded nine times: heptacosane $C_{27}H_{54}$ presumably derived from myristic acid appears five times. Nonacosane $C_{29}H_{60}$, M.P. 63–64°, was isolated by Pickles [1911] from the leaves and stems of bog myrtle and recently by Channon and Chibnall from cabbage. There also occurs nine times the description of triacontane $C_{30}H_{62}$, a hydrocarbon which alone of those described contains an even number of carbon atoms and which could not therefore be derived by the method postulated from any one single fatty acid. The evidence for the existence of this seems weaker than for that of any of the other hydrocarbons described and it appears not improbable that the so-called triacontane should really be

regarded as nonacosane, since the evidence rests chiefly on the determination of the melting point and this in all cases approximates closely to that of the nonacosane.

	M.P.	Source	
Nonacosane	63–64°	Bog myrtle	Pickles [1911]
	(uncorr. 62·7–62·8°)	Cabbage	Channon and Chibnall [1929]
Triacontane	64–65°	*Anthemis nobilis* flowers	Power and Browning [1914, 2]
	60–61°	Cotton-root bark	,, ,, [1914, 1]
		Euphorbia pilulifera	,, ,, [1913]
	63–65°	*Matricaria chamomilla* flowers	,, ,, [1914, 3]
	65·2°	*Eriodictyon* leaves	Power and Tutin [1907]
	65·5°	*Cluytia similis*	Tutin and Clewer [1912]
	65·5°	*Solanum angustifolium* leaves and flowers	,, ,, [1914]

Since Levene, West and van Scheer [1915] find that the melting-point of synthetic triacontane is 69–70°, the examination of the naturally occurring hydrocarbon which has been identified as triacontane needs revision; there are certainly grounds for believing that it may be identical with nonacosane.

If triacontane may be eliminated, the existence of hydrocarbons containing only odd numbers of carbon atoms agrees with their derivation from the fatty acids containing respectively 14, 15, 16 and 18 carbon atoms. The difficulty of obtaining the solid saturated acids from leaves in sufficient quantity to make a complete identification renders it difficult to say whether the acid with 15 carbon atoms does occur in the plant leaf. Acids with odd numbers of carbon atoms are described, as for instance, that with 21 carbon atoms which is several times recorded [*e.g.* Tutin and Clewer, 1912] and it may well be that α-oxidation of palmitic acid takes place in the plant leaf. The occurrence of dimyristyl ketone in the cabbage leaf together with the hydrocarbon derived from it by reduction is of great interest and suggests that the fatty acid containing 15 carbon atoms must occur at some stage in the metabolism of the leaf.

Summary.

1. The unsaponifiable matter of the spinach leaf contains in addition to carotene and a sterol, $C_{27}H_{46}O$, the hydrocarbon hentriacontane, $C_{31}H_{64}$, the identification of which has been confirmed by X-ray examination. Ceryl alcohol $C_{26}H_{54}O$ is also present. Palmitic, cerotic, oleic, linolenic acids and an isomeride of linoleic acid have been isolated.

2. The identification of nonacosane and *n*-dimyristyl ketone in the unsaponifiable matter of the cabbage leaf [Channon and Chibnall, 1929] is confirmed. The saturated fatty acids are palmitic and a higher fatty acid, probably behenic.

3. The question of the occurrence of the higher paraffin hydrocarbons in the leaf and their probable derivation is discussed.

We desire to acknowledge our gratitude to the Department of Scientific and Industrial Research for a grant which has given us the opportunity to

carry out this work and to express our thanks to Dr Piper for his kindness in carrying out the X-ray examination of the hydrocarbon.

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LXXI. THE CUPROUS DERIVATIVES OF SOME SULPHYDRYL COMPOUNDS.

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METALLIC derivatives of mercaptans have been long known and in the cases of mercury and lead extensively studied. The copper derivatives on the other hand, though they have been prepared on numerous occasions, have received comparatively little attention and have rarely even been analysed. It is one of the purposes of this paper to point out that the formation of a cuprous compound is a general reaction of mercaptans, and that such compounds are frequently very well suited for their isolation or purification.

Precipitates have frequently been obtained on adding a solution of a cupric salt to a mercaptan in neutral or slightly acid solution, but Klason [1877], using thioglycollic acid, first pointed out that it was a cuprous compound that was formed while some of the mercaptan was oxidised to disulphide. Before this Carius [1862] had prepared copper derivatives of mono-, di- and trithioglycerol; his analytical figures show that these contain univalent copper.

Suter [1895] and Gabriel [1905] as well as several more recent workers, have prepared mixtures of cuprous cysteine and cuprous chloride by adding copper sulphate solution to a solution of cysteine hydrochloride, while Mörner [1904] and Friedmann [1903] have investigated the cuprous derivatives of α- and β-thiolactic acids. It is probable also that the greyish-black precipitate which Lewis and McGinty [1922] obtained on adding copper sulphate to an extract of the urine of a rabbit which had been fed on phenylcarbamidocystine was the cuprous compound of phenylcarbamidocysteine.

So far the methods for the preparation of cuprous mercaptides have all involved the conversion of half the mercaptan to the corresponding disulphide, and this renders them unsuitable for preparing pure mercaptans. Hopkins's [1929] striking observation, that cuprous oxide would react with a mercaptan in acid solution to form the cuprous mercaptide, has changed the whole situation, since by this method, with a little care, a mercaptan may be quantitatively precipitated as its cuprous derivative and so obtained pure.

EXPERIMENTAL.

Part I. *The preparation of some cysteine and cystine derivatives.*

Acetylcysteine. Diacetylcystine was first made by Inouye [1929] by the hydrolysis of various diacetylcystine esters. Working at 37°, and adding the

theoretical amount of sodium hydroxide gradually so that the p_H never exceeds 9·5 (0·2 g. of glycine may conveniently be added to buffer the fluid at this p_H) I find that the hydrolysis of 5 g. of the methyl ester of diacetylcystine dissolved in 200 cc. of water takes 6 hours. The propyl ester, the use of which Inouye recommends, is much less soluble and the saponification of 5 g. suspended in 600 cc. of water requires 9 hours at 60°.

These methods are laborious and there is great risk of racemisation during the hydrolysis. A simpler method was therefore sought. Neither acetyl chloride nor acetic anhydride will react with cystine in pyridine suspension, but the latter, as Nicolet [1930] and Du Vigneaud and Hollander [1930] have also found, reacts smoothly in alkaline solution.

10 g. of cystine are dissolved in 50 cc. of 10 % sodium hydroxide, 16 cc. of acetic anhydride are added and the mixture is kept cool and stirred until homogeneous. The excess of acetic acid and acetic anhydride is removed by distillation at 40° *in vacuo* till a viscous gum remains. This is dissolved in 300 cc. of 4 % (by volume) sulphuric acid and reduced with 5 g. of zinc dust; the mixture is stirred occasionally and filtered after about 30 minutes. An aqueous suspension of cuprous oxide is cautiously added to the filtrate, care being taken that, after each addition, the precipitate has lost its initial pink colour before more cuprous oxide is added. The end-point is easily found by centrifuging a sample and adding cuprous oxide to the centrifugate, but, should it be overshot and the cuprous compound of acetylcysteine redissolve, the latter may be quantitatively re-precipitated by aeration. This phenomenon has already been described in the case of glutathione [Pirie, 1930].

The colourless cuprous compound is washed by repeatedly stirring up the precipitate in distilled water and centrifuging. After 3–4 washings the centrifugate remains cloudy even after 15 minutes at 3500 R.P.M. It is then sulphate-free and contains some dissolved cuprous acetylcysteine. On suspending the thoroughly washed solid in water and decomposing with H_2S (by the closed vessel method) a suspension of cuprous sulphide resembling strong coffee is obtained. This is perfectly transparent when diluted and the sulphide cannot be removed by centrifuging or filtering through kieselguhr. The solution is evaporated to complete dryness *in vacuo* at 45° and the residue is boiled up with glacial acetic acid. The cuprous sulphide may now be removed by filtration and the filtrate, after concentration by vacuum distillation, is evaporated to crystallisation in a desiccator over soda-lime. Whetstone-shaped crystals of acetylcysteine form readily on scratching the concentrated acetic acid solution with a glass rod.

The yield is variable, depending on the completeness of the precipitation with cuprous oxide but is generally about 6·5 g. M.P. (uncorr.) 107°–109°. (Found: N, 8·52 %; S, 19·9 %; Calc.: N, 8·59 %; S, 19·63 %.)

Chloroacetylcysteine. Fischer and Suzuki [1904] isolated dichloroacetylcystine by extracting with ether the solution obtained when chloroacetyl chloride is added to an alkaline solution of cystine. This method gives a pure

unhydrated product, but it is troublesome when used on a large scale. The hydrate, slightly contaminated with coloured products, will however crystallise from the acidified reaction mixture on standing for a day in the ice-chest.

To 22·3 g. of cystine, dissolved in 188 cc. of *N* NaOH, 16·7 cc. *i.e.* 25 g. of chloroacetyl chloride and 275 cc. of *N* NaOH are added concurrently in the course of an hour, the temperature being kept below 5° throughout. After standing at room temperature for 2 hours 34 cc. of concentrated hydrochloric acid are added and the mixture is filtered after being well shaken. It is put in the ice-chest and next day the hydrated dichloroacetylcystine is filtered off and washed with cold water. The yield at this stage is 27 g. and a further 3–5 g. of a less pure material may be obtained by distilling the filtrate and washings *in vacuo* until an oil separates, and cooling.

4 g. of hydrated dichloroacetylcystine and 1 g. of zinc dust are shaken at room temperature for an hour with 120 cc. of 2·5 % H_2SO_4. The dichloroacetylcystine and most of the zinc dissolve. Some H_2S is formed. If the reaction mixture is heated, or if a large excess of zinc is used, there is more of this destruction and some free hydrochloric acid, which interferes with the subsequent precipitation by cuprous oxide, is also formed. Dewar and Gamgee [1871] observed the production of H_2S during the reduction of cystine by tin, and some H_2S is almost always formed during the reduction of a disulphide by zinc even in the cold, but it is more marked in the case of dichloroacetylcystine than in that of any other compound that I have used.

The solution is filtered and, if necessary, air is blown through it to remove the H_2S; after warming to about 40° cuprous oxide suspension is added very gradually. When about half of the cuprous oxide has been added the action proceeds more rapidly and the mixture frequently sets to a perfectly rigid jelly which breaks up into a curd on stirring. Cuprous chloroacetylcysteine is soluble in presence of excess of cuprous oxide but is re-precipitated on aeration. Owing to the relative insolubility of chloroacetylcysteine in water it is unnecessary to wash the cuprous compound very thoroughly and a few washings with water on a Büchner funnel are adequate.

As in the case of acetylcysteine a colloidal solution of cuprous sulphide is formed if cuprous chloroacetylcysteine is decomposed with H_2S in aqueous suspension. When suspended in glacial acetic acid, however, especially in the presence of the electrolytes which were not removed by washing, the Cu_2S separates after a few hours as a precipitate. It is filtered and the filtrate, after concentration by distillation *in vacuo*, is evaporated in a vacuum desiccator over soda-lime. The crystals are recrystallised from water. Chloroacetylcysteine generally forms irregular angular masses but occasionally small regular octohedra are obtained. Yield 2–2·5 g. M.P. 126°. (Found: N, 6·95 %; S, 16·67 %; Cl, 17·34 %; Calc.: N, 7·09 %; S, 16·2 %; Cl, 17·97 %.)

Glycylcysteine. 4 g. of hydrated dichloroacetylcystine are put in a strong 100 cc. flask and 20 cc. of ammonia solution (sp. gr. 0·880) are added. Since the neutralisation of the acid by ammonia evolves considerable heat it is best

to add 5 cc. of ammonia first with cooling and then, when the solid has again reached room temperature, the remainder. The flask is tightly stoppered and kept in an incubator at 37° for 4 days. So far the method is simply a modification of that of Abderhalden and Spinner [1919] for the preparation of diglycylcystine.

As much of the excess ammonia as possible is removed by distilling the brownish-coloured solution *in vacuo* till it forms a gum adhering to the sides of the flask, this is dissolved in 20 cc. of 2 % sulphuric acid. To the acid solution 2·9 g. of finely powdered silver sulphate are added and the mixture is left, with occasional stirring, for some hours. It is important to precipitate all the chlorine at this stage. After filtering, 40 cc. of 2 % sulphuric acid are added to the filtrate, followed, after removing any excess of silver with H_2S, by 1–2 g. of zinc dust. Reduction is complete in about 20 minutes. The excess of zinc is filtered off and the filtrate heated to about 80° before any cuprous oxide is added. Precipitation will occur at a lower temperature if all the hydrochloric acid has not been removed or if a considerable amount of cuprous glycylcysteine has already been formed. If however cuprous oxide is added to a cold acid solution of glycylcysteine most of it is converted to cupric oxide and copper. As in the case of the two compounds already described an excess of cuprous oxide will cause re-solution of the already precipitated cuprous compound. On aeration it is re-precipitated as a bright yellow powder which, apart from its colour, is identical with the flocculent white mass which is formed at first.

The removal of the last traces of sulphuric acid from this substance is difficult, and as the properties of the resulting glycylcysteine show, I have probably never accomplished it quite completely. It is advisable to use a filtration method of washing, similar to that which has been described already [Pirie, 1930], rather than to wash by centrifuging. The cuprous glycylcysteine is somewhat unstable in the absence of acid and gradually becomes greenish on exposed surfaces when the washing has been carried nearly to completion. It is suspended in water and decomposed with H_2S. Decomposition is very rapid and after a few hours the solution may be filtered from cuprous sulphide. It is advisable to keep the volume of water used to suspend the cuprous compound as small as possible and to evaporate the filtrate (still containing some H_2S) rapidly in a vacuum desiccator over sulphuric acid and caustic soda. Residual sulphuric acid keeps the p_H of the resulting gum down to about 4 and makes crystallisation difficult. It will always crystallise however when seeded.[1] Owing to the excessive solubility of glycylcysteine in water the mother-liquor in contact with it is very viscous and filtration is slow. A

[1] After several early failures crystals were obtained by adding pyridine to the hydrochloric acid-containing gum which resulted when the treatment with silver sulphate was omitted. On washing with alcohol fairly pure glycylcysteine was obtained, but it is difficult to make the removal of pyridine hydrochloride by alcohol under these conditions complete. Since then I have experienced no difficulty with crystallisation.

further quantity of less pure material may be obtained by adding alcohol to the filtrate. As usual the yield is variable but 5–6 g. may be expected.

Glycylcysteine has no definite melting-point. It sinters at 130° and then gradually swells to a semi-solid foam which melts again at 177° and goes brown. (Found: N, 15·28 %; S, 18·08 %; Calc.: N, 15·73 %; S, 17·97 %.)

Methyl ester of acetylcysteine. The disulphide is prepared by a method based on that of Inouye [1929]. A suspension of 6·8 g. of cystine dimethyl ester hydrochloride in 50 cc. of dry pyridine is cooled to 0° and 4 cc. (excess) of acetyl chloride added gradually. Considerable heat is developed and the ester hydrochloride dissolves when about half the acetyl chloride has been added; crystals separate on adding the remainder. After standing at room temperature for 3 hours it is filtered through a small Hirsch funnel. Great care must be exercised here since the white crystals which remain on the funnel are apt to turn brown and melt, and occasionally ignite, on exposure to air. As soon as the fluid has been sucked through the funnel is removed from the flask and washed out. I have attempted to avoid this by adding the theoretical amount of acetyl chloride, but this results in a very much diminished yield. The filtrate is evaporated in a vacuum desiccator over sulphuric acid till crystallisation commences. On mixing with its own volume of water a mass of crystals is obtained and filtered off. The filtrate is brought to about p_H 6 with 10 % sodium hydroxide; on removing most of the liberated pyridine in a vacuum desiccator further crystallisation takes place. The crystals are washed with cold water.

5·9 g. of colourless crystals, M.P. 128–9°, are obtained. Unlike Inouye, I find that the dimethyl ester of diacetylcystine is perfectly stable in air and not at all hygroscopic. (Found: N, 7·95 %; S, 18·19 %; Calc.: N, 7·95 %; S, 18·18 %.)

0·380 g. was made up to 10 cc. with water and the rotation measured in a 19·95 cm. polarimeter tube.

Using the two mercury lines the rotations were $-7{\cdot}35°$ and $-6{\cdot}53°$ these values gave $[\alpha]^{17}_{577} = -96{\cdot}94°$ and $[\alpha]^{17}_{546} = -86{\cdot}13°$. The latter figure is in good agreement with the value ($-85{\cdot}59°$) given by Voss, Guttmann and Klemm [1930].

Zinc dust is added to 2 g. of diacetylcystine dimethyl ester dissolved in 150 cc. of 2 % sulphuric acid in the cold. The solution is filtered and treated with cuprous oxide at about 30° in the usual way. After washing, the cuprous acetylcysteine ester is suspended in glacial acetic acid and decomposed with H_2S. The cuprous sulphide remains colloidal even on boiling so the suspension is evaporated to dryness *in vacuo* and boiled with benzene. The benzene solution is filtered and evaporated in a desiccator over paraffin after most of the benzene has been removed on a water-bath. The residue may be recrystallised from water containing a little acetic acid and melts at 80°. (Found: N, 7·94 %; S, 18·35 %; Calc.: N, 7·91 %; S, 18·08 %).

Diacetylcystine dipropyl ester. Propyl alcohol is dried by distilling from lime and 6 g. of cystine are suspended in a litre. Hydrogen chloride is passed

into the mixture, boiling under a reflux condenser, for 2 hours. It is filtered hot and evaporated to small bulk by vacuum distillation. 6 g. (M.P. 178–9°) of cystine dipropyl ester hydrochloride separate out on cooling with ice. A further crop of crystals separates on adding ether.

7 g. of ester hydrochloride are acetylated in 100 cc. of pyridine with 4·2 cc. of acetyl chloride. After lying overnight in the ice-chest the solution is filtered and the filtrate distilled *in vacuo* to remove most of the pyridine. On adding ether 5·9 g. of diacetylcystine dipropyl ester separate. On recrystallising from water masses of fine needles are formed which are not hygroscopic and melt at 124–5°. (Inouye gave 175° and 117–8° as the melting-points of cystine dipropyl ester hydrochloride and diacetylcystine dipropyl ester respectively.) (Found: N, 6·51 %; S, 15·55 %; Calc.: N, 6·86 %; S, 15·69 %.)

Dicinnamylcystine dimethyl ester. 12 g. of cinnamyl chloride are added in the course of 5 minutes to a cooled suspension of 10·5 g. of cystine dimethyl ester hydrochloride in 200 cc. of dry pyridine. The mixture is then left for 8 hours at room temperature. The pyridine is distilled off *in vacuo* till crystals separate. These are filtered off, after standing overnight in the ice-chest, and the filtrate is poured into water. Both the crystals and the solid which separates when the pyridine filtrate is poured into water are recrystallised from alcohol. The product crystallises in very pale yellow leaflets which melt at 161–2°. (Found: N, 5·06 %; S, 12·21 %; Calc.: N, 5·30 %; S, 12·12 %.)

0·3024 g. of dicinnamylcystine dimethyl ester are dissolved in acetic acid and made up to 10 cc. At 17° the rotations for the mercury lines are $-1{\cdot}21°$ and $-0{\cdot}82°$ in a 2 dm. tube, *i.e.* $[\alpha]^{17}_{577} = -20°$ and $[\alpha]^{17}_{546} = -13{\cdot}6°$.

Oxidised glutathione. Hopkins [1929] observed that when glutathione was oxidised by aeration at p_H 7·6 in the presence of a trace of iron, the product isolated, after adding sulphuric acid to remove the baryta used to adjust the p_H, by evaporation followed by treatment with alcohol, had a low nitrogen and sulphur content. Very slow oxidation in the absence of iron was found to give similar results so other oxidising agents were tried. Hydrogen peroxide proved to be very much the most suitable.

A 10–20 % solution of glutathione is used and exactly the theoretical amount of 30 % hydrogen peroxide is added. Copper catalyses the oxidation of the —SH group at this p_H (2·8) and in the presence of 1 mg. of copper the oxidation of 10 g. of reduced glutathione is complete in 3 hours. So long as an excess of hydrogen peroxide is not added no side reactions occur and 1 mol. of H_2O_2 will cause the complete disappearance of the nitroprusside reaction from a solution containing 2 mols. of glutathione. Glutathione prepared from its cuprous compound may contain less than 1 part of copper per million. It is generally necessary therefore to add a trace of copper sulphate or oxide. When oxidation is complete the solution is cooled to 0°, put in a vacuum desiccator, and exhausted rapidly so that it freezes. The vacuum is maintained and in the course of a day all the ice evaporates leaving the oxidised glutathione as a white feathery non-hygroscopic mass easily soluble in water. It has not

yet been crystallised. Oxidised glutathione prepared in this way gives analytical figures for N and S identical with those of the reduced glutathione used in its preparation.

Mason [1931, 2] in a very recent paper has pointed out that oxidised glutathione holds alcohol tenaciously. It is obvious therefore that, apart from its very great convenience, the method of preparation of oxidised glutathione just described has no merit except the avoidance of alcohol. The presence of alcohol explains Pirie and Pinhey's [1929] observation that the oxidised glutathione used contained 17 % of an impurity which had no titratable groups.

Part II. *Preparation and properties of the cuprous derivatives of some thiol compounds.*

Cysteine. Although mixtures of cuprous cysteine and cuprous chloride have been prepared by several different workers, no one appears to have prepared pure cuprous cysteine.

l-Cystine (*dl*-cystine behaves in a substantially similar way though the cuprous compound of *dl*-cysteine is rather more soluble in weak acid than that of *d*-cysteine) is dissolved in 2 % sulphuric acid and some zinc dust is added. The solution is boiled and filtered. Both titration with iodine and polarimetric observation indicate that 97–100 % of the cystine has been reduced.

Cuprous cysteine is not readily precipitated from this solution in the cold. A reddish or grey precipitate is obtained and a considerable amount of copper sulphate is formed. Precipitation occurs smoothly in the cold if a trace of hydrochloric acid or a chloride is present, but in the absence of chloride it will take place at the boiling point. Being fairly soluble in boiling dilute sulphuric acid, cuprous cysteine may remain dissolved, but it precipitates on cooling. It is soluble in 5 % sulphuric acid even in the cold, but shows no sign of being soluble in presence of excess of cuprous oxide.

For analysis it is washed a little on the centrifuge and then transferred to a small Jena glass filter of the type used in micro-analysis. Washing is continued, with suction, until the wash-water is sulphate-free or until the cuprous compound begins to turn green. The latter happens first in the case of cysteine. The filter is put in a desiccator over P_2O_5 at room temperature and exhausted to 1 mm. A hard cake is obtained in this way which is much less readily oxidised by air than the loose mass which results if the precipitate in the centrifuge tube is dried.

A sample, containing about 6 mg. of copper, is transferred to a weighed 100 cc. Kjeldahl flask, with all but 8 cm. of its neck cut off, and incinerated with 0·5 cc. of sulphuric acid and some potassium sulphate. When cold, saturated sodium carbonate solution is added till the solution is alkaline. It is then acidified with acetic acid and, after the addition of some solid potassium iodide, titrated with $N/100$ thiosulphate using starch as indicator. If the incineration has been thorough the end-point is perfectly sharp. The

ordinary Pregl method of analysis for copper has been found not to work satisfactorily with many of these compounds.

21·1 mg. of the dried material required 11·1 cc. of $N/98$ thiosulphate whence, Cu, 34·2 %; Calc. Cu, 34·6 %.

Like other compounds of this type, cuprous cysteine is soluble in dilute HCl and in alkalis, it is also soluble in unusually dilute sulphuric acid, for most cuprous compounds are not appreciably soluble in sulphuric acid weaker than 20 %.

Acetylcysteine and chloroacetylcysteine. The cuprous derivatives of these two substances are similar in their general properties. Both are soluble in presence of excess of cuprous oxide and in moderately concentrated (10–20 %) sulphuric acid. They are also soluble in water in the absence of acid; thus on washing a sample by centrifuging, the p_H of each successive centrifugate is higher till a p_H of about 3·6 is reached. At this point the compound goes into solution and the wash-water is found to be sulphate-free. A saturated solution of cuprous acetylcysteine at p_H 3·6 contains 44 mg. of copper per 100 cc. On adding a little sulphuric acid, mixing rapidly and allowing the mixture to stand, a rigid jelly is obtained in about 15 minutes. The concentration of cuprous acetylcysteine in this jelly is 0·15 % so that it shows a gel-forming capacity of the same order as that of dibenzoylcystine [Wolf and Rideal, 1922]. Cuprous chloroacetylcysteine is less soluble in water (it forms a solution of about 0·07 %) and, although it also will form remarkably rigid jellies, gel-formation is not so reliable as with cuprous acetylcysteine.

When re-precipitated from solution cuprous acetylcysteine does not dry readily and tends to blacken. As a result the analytical figures are generally low: *e.g.* directly precipitated; different preparations contained 27·9, 27·5 and 28·1 % Cu: re-precipitated; different samples contained 25·1, 22·1, 25·0, 24·8 and 26 % Cu; Calc. for $C_5H_8O_3NSCu$ 28·2 % Cu.

Cuprous chloroacetylcysteine also is not very stable on drying and always gives rather low analytical figures. (Found: 23·1 % Cu; Calc.: 24·5 % Cu.)

The p_H of an aqueous solution of cuprous acetylcysteine gradually rises on exposure to air and may reach 7. At this stage nothing is precipitated by acid and the fluid will dissolve further quantities of solid with consequent fall in p_H. It is probable that this rise in p_H is due to the oxidation of cuprous copper to the cupric form. This then combines with the carboxyl instead of with the sulphydryl group. A titration curve indicated that, while there was a group with a p_K in the neighbourhood of 4, there was no other titratable group with a p_K less than 12. This suggests that, as would be expected, the copper in cuprous acetylcysteine is linked to the sulphur atom.

If either compound is dissolved in 2 % sulphuric acid and put in a narrow-mouthed vessel, with a stream of hydrogen passing through to prevent oxidation, the initially formed cuprous compound is completely redissolved on the gradual addition of two atomic proportions of copper, in the form of cuprous oxide suspension of known strength. If too little cuprous oxide is

added, traces of the normal cuprous compound remain undissolved, if too much is added metallic copper deposits. The solution formed is yellow or brown. For every 10 cc. of solution 100 cc. of 97 % alcohol are put into a Büchner flask and the solution is filtered directly into it through a previously moistened paper. A yellowish gelatinous precipitate is formed but it is too unstable to give consistent analytical figures; probably, by analogy with glutathione, it is a complex of two molecules of cuprous acetylcysteine and one of cuprous sulphate.

Glycylcysteine. Like cysteine this substance forms a cuprous compound in the cold in the presence of a chloride, but in the absence of chloride it is necessary to heat to 80° before adding the cuprous oxide. It resembles cysteine also in forming a cuprous compound from which the sulphuric acid is not readily removed by washing. It is perfectly stable when dried. (Found: 27·36 %, 26·77 % and 26·6 % Cu; Calc.: 26·44 % Cu.)

Cuprous glycylcysteine reacts readily with more cuprous oxide when it is suspended in 6 % sulphuric acid in the absence of air. The product obtained by filtering the solution into alcohol and then washing the precipitate with alcohol till it starts to turn green is stable when dry. (Found: Cu, 30·7 %; SO_4, 23·06 %

Calc. for $(C_5H_9O_3N_2SCu)_2Cu_2SO_4$ Cu 36·1 %, SO_4, 13·63 %.
Calc. for $C_5H_9O_3N_2SCu.CuSO_4$ Cu 31·7 %, SO_4, 24·0 %.)

It is thus apparent that the analytical figures are in much better agreement with a formula in which there is one atom of cupric copper than with one in which both are cuprous. Nevertheless, the fact that re-solution of cuprous glycylcysteine occurs on the addition of one atomic proportion of cuprous copper in absence of air and without any free copper being formed, taken in conjunction with the fact that cuprous glycylcysteine is re-formed on aeration (analysis of the yellow material shows that it contains 26·7 % Cu), seems to me to indicate that both atoms of copper are in the cuprous state. Sulphuric acid is a probable contaminant in view of the necessarily incomplete washing, although 9·43 % is undoubtedly more than would have been anticipated.

Methyl and propyl esters of acetylcysteine. Both react normally in acid solution and give cuprous derivatives which dissolve on the addition of another atomic proportion of cuprous copper. So far I have been unable to isolate these double copper compounds since they are not only soluble in alcohol but are also very readily autoxidisable.

Propyl acetylcysteine was prepared from the disulphide by suspending it in 2 % sulphuric acid at about 50° and adding zinc dust. The disulphide is rather difficult to wet but it is reduced in about half an hour. The cuprous derivatives of both these compounds are insoluble in water but tend to remain in suspension in water when they have been washed free from acid. The cuprous sulphide suspensions formed on decomposing either of these with

H_2S are remarkably stable and, if free from electrolytes, will withstand boiling with alcohol or glacial acetic acid. Acetylcysteine propyl ester has not been obtained crystalline.

Both cuprous compounds are stable on drying:

$CH_3CONHCH(COOCH_3)CH_2SCu$	Found; 26·00 % Cu; Calc. 26·55 % Cu.
$CH_3CONHCH(COOC_3H_7)CH_2SCu$	Found; 23·2 % Cu; Calc. 23·77 % Cu.

Glutathione. Cuprous glutathione has been more extensively studied than the other cuprous compounds. Hopkins [1929] observed that it redissolved in the presence of an excess of cuprous oxide. For some reason it will not re-precipitate on aeration when an excess of cuprous oxide has been added to a solution recovered from a mercury precipitate from a boiled yeast extract, whereas, as I have already pointed out [Pirie, 1930], it may be re-precipitated from a yeast filtrate prepared directly. This is due, in part at any rate, to the formation of interfering substances (cysteinylglycine?) on boiling and to their concentration in the filtrate from mercuric sulphate. A certain amount of re-precipitation is obtained if such a solution is largely diluted with water and aerated.

On dissolving glutathione in dilute sulphuric acid and adding cuprous oxide in a current of hydrogen to exclude air, maximum precipitation is obtained after the addition of one atomic proportion of copper. The addition of a second atom of copper results in complete re-solution, but a third may be added before any turbidity due to metallic copper appears. A white precipitate is obtained if the filtrate from this mixture is allowed to run into alcohol. On washing this precipitate it turns slightly green but seems to be stable.

The analytical figures obtained depend on the thoroughness with which it is washed with alcohol. On very thorough washing: Cu, 25·5 % and 25·45 %; SO_4, 14·2 % and 14·9 %; Calc. for $[(CuSG)_2Cu_2SO_4]_2SO_4$:[1] Cu, 25·1 % and SO_4, 14·25 %. With less thorough washing the figures are 26·44 % and 16·13 % respectively.

In this case it is obvious that all the extra copper cannot be in the cupric state since there is not enough sulphate present. The figures show further that there are only two atoms of copper present for each atom of sulphur whereas three may be added without any deposition of copper occurring. It is noteworthy that products having the same analytical figures are obtained irrespective of whether two or three atomic proportions of copper have been used to dissolve the glutathione. In the latter case there is some copper in solution in the alcohol. Probably therefore a compound of the type $CuSG.Cu_2SO_4$ is present in aqueous solution, but on pouring the solution into alcohol it is broken down. In view of the extreme difficulty experienced in washing all the sulphuric acid out of certain cuprous compounds, *e.g.* that of glycylcysteine,

[1] GSH is reduced glutathione.

it seems probable that the substance actually precipitated by alcohol is $(CuSG)_2Cu_2SO_4$ and that the extra sulphate is adventitious.

The behaviour of the soluble copper compound towards carbon monoxide is interesting. Joannis [1903] showed that sulphuric acid saturated with carbon monoxide would dissolve finely divided copper and isolated Cu_2SO_4, 2CO, H_2O. If, without admitting any air, the hydrogen which was used to exclude air during the solution of cuprous glutathione in an excess of copper oxide, is replaced by a stream of carbon monoxide, precipitation of cuprous glutathione occurs. Precipitation is nearly complete in 3 hours. At this stage, if the CO is displaced by a current of hydrogen, there is complete solution of the precipitate. On passing in more CO precipitation occurs again, and so on. The behaviour of CO is thus entirely different from that of oxygen and seems to involve the reaction:

$$(CuSG)_2Cu_2SO_4 + 2CO \rightleftharpoons 2CuSG + Cu_2SO_4, 2CO.$$

If the suspension obtained by saturating with carbon monoxide is centrifuged, with a stopper in the centrifuge tube to prevent too much loss of CO, and the centrifugate is exhausted until it boils and then left for a day with occasional re-exhaustion, a precipitate of copper is formed. The fluid, which initially liberated very little iodine from potassium iodide, now liberates a large amount. This behaviour is what would be expected if the centrifugate contained the carbon monoxide complex of cuprous sulphate.

Glycylcysteine anhydride. Oxidised glutathione, prepared by the method already described, is boiled in water to form the diketopiperazine by the method of Hopkins [1929]. The resulting disulphide is reduced with zinc. Towards cuprous oxide the reduced product behaves exactly like glycylcysteine except that it is unnecessary to heat the solution to more than 30° before adding the cuprous oxide. The cuprous compound turns greenish when washed and is not very stable. (Found: Cu, 28·48 %; Calc.: Cu, 28·57 %.)

In acid solution the cuprous compound is soluble in presence of an excess of cuprous oxide but it does not appear to stabilise a whole molecule of cuprous sulphate as does glutathione although it might have been expected to do so since it also has two peptide linkages. The gelatinous product obtained on pouring this solution into alcohol is not sufficiently stable to give consistent analyses.

Methyl ester of cinnamylcysteine. Since the dimethyl ester of dicinnamylcystine is insoluble in water it is reduced with zinc in glacial acetic acid containing a few drops of concentrated sulphuric acid per 100 cc. The solution is boiled, filtered and then, when again boiling, treated with cuprous oxide in aqueous suspension. A yellowish curd separates which is washed with acetic acid and then with water till sulphate-free. (Found: Cu, 18·9 %; Calc. for $C_{13}H_{14}O_3NSCu$: Cu, 19·4 %.)

A suspension of cuprous sulphide formed on decomposing this, suspended in glacial acetic acid, with H_2S but it filtered after being boiled. A white curd

separated on adding water to the concentrated acetic acid solution. (Found: N, 4·81 %; S, 11·50 %; Calc. for $C_{13}H_{15}O_3NS$: N, 5·3 %; S, 12·12 %.)

It gives a nitroprusside reaction and, when oxidised, decolorises an acetic acid solution of bromine. On pouring this solution into water a curd containing 13 % of bromine is obtained. It is therefore improbable that the reduction has saturated the double bond.

Benzoylcysteine. This compound has not been obtained pure. Dibenzoylcystine is shaken with zinc in a mixture of equal volumes of 2 % sulphuric acid and alcohol. It is advisable to add a trace of copper sulphate to catalyse the action. If necessary the mixture is warmed but as a rule all the dibenzoylcystine dissolves easily. After filtering from undissolved zinc and removing the alcohol by distillation *in vacuo*, an oil separates which does not solidify on cooling. This is washed by boiling up again with water and dried in a vacuum. It remains viscous.

Equal volumes of 3 % sulphuric acid and alcohol are used to dissolve this gum and a trace of sodium chloride is added. On adding cuprous oxide to the hot fluid a fawn-coloured precipitate is formed. There is no sign of this being soluble in presence of excess of cuprous oxide. For analysis it is suspended in water in an atmosphere of hydrogen, and dilute sodium hydroxide solution is added. The suspension is centrifuged and the centrifugate poured into dilute sulphuric acid. There is no sign of gel formation and the white precipitate is washed and dried as usual. (Found: Cu, 21·4 %; Calc. for $C_{10}H_{10}O_3NSCu$: Cu, 22·1 %.)

When suspended in alcohol and decomposed with H_2S the cuprous sulphide is not colloidal though it is when suspended in acetic acid. Benzene precipitates benzoylcysteine from its concentrated alcoholic solution as a stringy mass and I have not yet obtained it in satisfactory form.

Cysteinylglycine. Mason [1931, 1] showed that cysteinylglycine did not form a cuprous compound insoluble in sulphuric acid. The compound, prepared by the method of Kendall, Mason and McKenzie [1930], dissolves cuprous oxide in acid solution. There is no precipitation on adding sodium hydroxide till the p_H is about 4, but on evaporating this brown fluid *in vacuo*, a grey precipitate is formed. On exposing this to air it immediately turns chocolate in colour. This abnormal behaviour of cysteinylglycine is difficult to understand, but further investigation of it is in progress. Preliminary work shows that the benzoyl derivative of cysteinylglycine forms a cuprous compound of the usual character.

Cysteine methyl ester. The amount of saturated silver sulphate solution necessary to combine with all the hydrochloric acid is added to some cystine dimethyl ester hydrochloride together with the same volume of 2 % sulphuric acid. The mixture is centrifuged and some zinc is added, after about 10 minutes it is filtered. To the filtrate, cuprous oxide suspension is added, air being excluded by a current of hydrogen. No insoluble cuprous compound is formed, but the copper oxide dissolves with the formation of a perfectly colourless

solution. Two atomic proportions of copper may be added in this way but a third results in a pale green colour and a faint turbidity.

When poured into alcohol a white precipitate forms and the alcohol takes on a bluish tinge. The precipitate rapidly turns grey and does not give consistent analyses. It appears however to have the composition

$$[CuSCH_2CH(COOCH_3)NH_2]_2SO_4.$$

The alcohol contains more copper than the precipitate. This indicates that, as in the case of glutathione, the more complex copper compound is unstable in alcohol.

Discussion.

Several reasons for the formation of soluble cuprous compounds suggested themselves at the beginning of this work but all have been excluded except one.

A carboxyl group cannot be concerned since the methyl ester of acetylcysteine and glycylcysteine anhydride both dissolve when an excess of cuprous oxide is present.

An amino-group cannot be concerned since acetylcysteine, chloroacetylcysteine, acetylcysteine methyl ester and glycylcysteine anhydride all form soluble compounds.

It is improbable that subsidiary valencies of the sulphur atom are concerned since cysteine and thioglycollic acid do not form soluble compounds. (The cuprous compounds of thiourea and thioglycollic acid have been prepared by several different workers; I find that neither will dissolve on adding an excess of cuprous oxide.)

The peptide linkage remains as a possibility and it seems to be a highly plausible one. Cuprous benzoylcysteine it is true does not dissolve in presence of an excess of cuprous oxide; but benzoylcysteine itself is insoluble in dilute acids unlike the other compounds used. A more serious objection is that none of the sulphur-free peptides which I have tried (*i.e.* glycylglycine, diglycylglycine and leucylglycylglycine) will stabilise cuprous oxide in dilute sulphuric acid. It seems therefore that, if the theory is correct, the copper mercaptide linkage in the molecule is a necessary precursor of any further action.

The examples given in this paper should serve to show the value of the cuprous compound as a means of separating mercaptans from the other products of a reaction and of getting a pure product without having to recrystallise. Different mercaptans cannot of course be easily separated from each other, though in some cases, *e.g.* a mixture of cuprous acetylcysteine and cuprous glycylcysteine, it is possible to concentrate the two compounds in different fractions.

With the exception of some purines, mercaptans seem to be the only compounds which form cuprous derivatives insoluble in acid solution. A purine-like body, containing 30–36 % of nitrogen, has in fact been isolated from a yeast extract, prepared in the way already described [Pirie, 1930], by adding a large amount of cuprous oxide to the fluid after as much glutathione as possible

has been removed. A yellowish precipitate forms which on washing and decomposing with H_2S gives a white solid soluble in acids, alkalis and H_2S solution but insoluble in water. It has not been further investigated.

In view of the solubility of the cuprous derivatives of cysteine methyl ester and cysteinylglycine and of the difficulty experienced in washing cuprous glycylcysteine or cuprous cysteine free from sulphuric acid, it is advisable to work with acidic rather than with neutral or basic compounds. When dealing with an unknown sulphydryl compound it would therefore be wise to acetylate or benzoylate an amino-group before proceeding to cuprous precipitation.

Attempts to utilise the specificity of this cuprous compound precipitation in a method for the estimation of glutathione have so far been unsuccessful. The most hopeful results were obtained when a tissue extract, warmed to 50–60°, was titrated with a mixture of copper sulphate and sodium hypophosphite. A precipitate was obtained and, on adding an excess of the reagent, it redissolved giving a fairly sharp end point. The figures obtained were however always very high and were much less reliable than those obtained by iodimetric methods.

The specific rotations of the sulphydryl compounds prepared have not been given in the text since they are unreliable, partly because of the possibility that some of the disulphide is present and partly because the observed angle is so small. The following values for $[\alpha]^{17}_{5461}$ were observed in acid solution:

Acetylcysteine + 0·42°; chloroacetylcysteine + 8·7°; glycylcysteine + 6·9°; methyl ester of acetylcysteine − 31·0°.

In describing the effect of carbon monoxide on the cuprous sulphate complex of a copper mercaptide only glutathione has been mentioned. All the other sulphydryl compounds which form soluble cuprous derivatives with an excess of cuprous oxide, but insoluble derivatives with one atom of copper, behave in precisely the same way.

Summary.

Acetylcysteine, chloroacetylcysteine, glycylcysteine, the methyl ester of acetylcysteine, and the dimethyl ester of cinnamylcysteine have been prepared and the method of preparation is given in each case.

Improvements are suggested in the preparation of oxidised glutathione, dichloroacetylcystine and the dimethyl and dipropyl esters of diacetylcystine.

Cinnamylcysteine methyl ester, acetylcysteine propyl ester and benzoylcysteine have been prepared in an impure state.

The cuprous derivatives of these compounds have been prepared and complexes of these derivatives with cuprous sulphate have in many cases been isolated and analysed. These complexes are decomposed reversibly by carbon monoxide and irreversibly by oxygen.

The suitability of cuprous derivatives for the isolation and purification of sulphydryl compounds is emphasised.

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LXXII. THE ACTION OF DYESTUFFS ON ENZYMES.

I. DYESTUFFS AND OXIDATIONS.

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During the past year, a series of investigations has been carried out in this laboratory on the toxic action of dyestuffs on enzymes and on the catalytic activities of tissues. About thirty dyestuffs of various types have so far been examined and it is already clear that not only do the dyestuffs vary very widely amongst themselves in their attack upon enzymes but that the enzymes exhibit a considerable specificity of behaviour towards the dyestuffs. Marston [1923] showed that the proteolytic enzymes are specifically precipitated by dyes of the safranine type, the compounds produced still being proteolytically active. In the investigations to be described, however, the main object has been to determine the amount of poisoning action a dyestuff has upon an enzyme, and how far the union of dyestuff and enzyme is reversible. Whilst this work was in progress, Bamann and Schmeller [1931] published a paper showing that substances of the indicator type, *e.g.* phenolphthalein, bromothymol blue, *etc.*, have a toxic action on lipase and there is little question but that a study of the action of these substances and of dyestuffs, such as those which are discussed in this paper, will lead to valuable information bearing on the constitution of enzymes.

The fact that dyestuffs have specific toxic actions which can be accurately estimated is a very great aid in distinguishing between enzymes which are closely related and between which there appears at present to be no satisfactory method of differentiation. Moreover, as will be shown subsequently, it is possible, by making use of the knowledge that certain dyestuffs have toxic actions upon an enzyme which is normally intracellular, to gain information on the permeability of cells (*e.g.* bacteria) to these dyestuffs.

The enzymes at present under investigation are those concerned with oxidations and reductions and also with hydrolytic changes, *e.g.* fumarase, urease, diastase.

The present communication is concerned with the effects of dyestuffs upon the oxidations of a number of substrates by bacteria (*B. coli*), and by muscle and brain tissues, succinate in particular being investigated.

Experimental.

The method, in brief, was to determine the amount of oxygen consumed in a certain time by a suspension of bacteria, or a quantity of muscle or brain tissue, in presence of a substrate, and to compare this with the oxygen consumed after the bacteria or tissue had been exposed to the action of a dyestuff (usually at a concentration of 1/5000) for 30 minutes at 37°. The consumption of oxygen was measured in the Barcroft differential manometer, all experiments being carried out at 37°.

There were placed in each vessel of the apparatus 1 cc. of a suspension of bacteria, 1 cc. of $M/5$ phosphate buffer, p_H 7·4, and 0·5 cc. of a 1/1000 solution of the dyestuff. The apparatus was shaken at 37° at the usual rate for half an hour, the taps of the vessels being open. After this interval, 0·5 cc. of a solution of the substrate under investigation was placed in the right hand vessel and 0·5 cc. of saline in the left hand vessel. The taps were closed after a few minutes' further shaking to secure temperature equilibrium, and the rate of oxygen uptake was measured. A control experiment, in which water was substituted for the dyestuff, was carried out in every case. The rates of oxidation were approximately linear, and the oxidation was allowed to proceed for 2 hours (or a less period if this proved more convenient). The oxygen uptakes in a series of experiments at the end of this period were measured and the percentage inhibition effected by the dyestuff calculated. To give an example, the oxygen taken up in $1\frac{1}{2}$ hours at 37° by 1 cc. of a suspension of *B. coli*, unexposed to dyestuff, in presence of 0·067 *M* sodium lactate was 1367 mm.^{3} The oxygen uptake by the same amount of the suspension of *B. coli* under the same conditions but after exposure to Congo red (1/5000) was 1161 mm.^{3}, and after exposure to ethyl violet (1/5000) 201 mm.^{3} The percentage inhibition in the first case was 15 and in the second case 85.

The acid substrates were used in the form of their sodium salts, the p_H of solutions being 7·4.

The *B. coli* suspension was prepared by 48 hours' growth in tryptic broth, the organism being centrifuged from the broth and washed twice with normal saline. It was made into a thick suspension in normal saline and stored at 0°.

In the cases of muscle and brain, 0·5 g. of the tissue was used. Muscle, after dissection from the animal, was cooled and minced. Brain was similarly treated, the whole brain being used.

Choice of dyestuffs.

A fairly representative series of dyestuffs has been chosen. This includes 10 members of the triphenylmethane series (malachite green, brilliant green, methyl violet, crystal violet, ethyl violet, gentian violet, acid green, acid fuchsine, water blue, soluble blue), 3 monoazo-dyestuffs (orange G, chrysoidine, and crystal scarlet) and 3 tetrazo-dyestuffs (Congo red, benzopurpurine, and

Bismarck brown). There is 1 diphenylmethane dyestuff, auramine. The pyronine series is represented by pyronine, eosin and erythrosin; the thiazines by methylene blue and toluidine blue; the safranines by safranine and methylene violet; and the eurhodines by neutral red and Janus green. There are also included acriflavine, haematoxylin, and an anthocyanin, cyanin chloride. It is proposed later to add to this series, but it has been felt that there are sufficient at present under investigation to indicate the main features of the action of dyestuffs on enzymes.

Dyestuffs and oxidations by B. coli.

The results of the action of dyestuffs on the oxidations by *B. coli* of glucose, lactate, succinate and formate are shown in Table I, the final concentrations of these substrates in the Barcroft vessels being 0·017 *M*, 0·067 *M*, 0·067 *M*, and 0·05 *M*, respectively.

It will be seen that, under the experimental conditions employed, whereas 15 of the dyestuffs have a relatively large inhibitory action on the oxidation by *B. coli*, the remaining 14 have little or none.

With regard to the toxic dyestuffs it may be imagined, in the first instance, that the toxicity of the dyes to the oxidations of *B. coli* is due primarily to the lethal action of these substances. Previous evidence, however, has shown with certainty that lethal action is not necessarily connected with a poisoning action on the oxidative mechanisms of the cell. For instance, toluene, which has a lethal action on *B. coli*, does not inhibit the oxidative action of this organism on succinate, lactate or formate [Quastel and Wooldridge, 1927]. Again ultra-violet light which induces great mortality among the cells of *B. coli* has little action on the oxidation by this organism of glucose or lactate [Cook and Stephenson, 1928]. It would be unreasonable, therefore, to ascribe the action of the dyestuffs which inhibit the oxidations of *B. coli* to a lethal action of these substances. An analysis of the results shown in Table I bears out this conclusion. Firstly it will be seen that in many cases the oxidation of lactate or glucose is affected to a greater extent than that of succinate. If a lethal action were responsible, such difference in effects would not be anticipated. Secondly, a specificity of behaviour is observable, for whereas one dyestuff will inhibit lactate oxidation much more powerfully than that of succinate, another will inhibit both to the same extent. Such specificity of behaviour would not be expected if the dyestuffs acted solely by a lethal action.

It is more reasonable to suppose that the toxic action of the dyestuffs is due to their combination with certain enzymes involved in the oxidations. It would be expected, if this were true, that the dyestuffs which are toxic would also be capable of staining the cell and this actually is the case. The reverse conclusion, however, *i.e.* that a dyestuff capable of staining the cell is necessarily toxic to oxidations, does not follow; for (*a*) the combination of enzyme

Table I. *Percentage inhibitions by dyestuffs of oxygen uptakes by* B. coli *in presence of various substrates.* $p_H = 7{\cdot}4$. Temp. 37°.

Dyestuff (1/5000)	Nature of dyestuff	Glucose	Lactate	Succinate	Formate
Malachite green	Basic	100	92	72	71
Brilliant green	,,	68	66	67	46
Methyl violet 6 B	,,	100	88	46	86
Crystal violet	,,	97	91	53	92
Ethyl violet	,,	82	85	69	84
Gentian violet	,,	98	80	36	75
Methylene blue	,,	57	33	75	28
Toluidine blue	,,	46	38	72	24
Auramine	,,	93	87	51	54
Acriflavine	,,	100	95	45	58
Neutral red	,,	92	72	37	50
Janus green	,,	100	69	29	45
Chrysoidine	,,	95	34	19	44
Pyronine	,,	39	20	6	17
Safranine	,,	98	86	22	52
Methylene violet	,,	19	5	10	25
Bismarck brown	,,	0	4	8	27
Acid fuchsine	Acid	0	0	2	0
Acid green	,,	0	17	3	0
Soluble blue	,,	0	0	5	9
Water blue	,,	0	8	7	0
Orange G	,,	0	4	2	14
Crystal scarlet	,,	1	0	7	16
Congo red	,,	0	15	10	0
Benzopurpurine	,,	0	31	22	22
Eosin		4	21	2	3
Erythrosin		6	18	16	9
Haematoxylin		17	8	39	35
Cyanin chloride		15	12	14	13

and dyestuff may not be such as to prevent activation of the substrate occurring and (*b*) the dyestuff may combine with structures in the cell which are not connected with the enzymes under consideration. It may be pointed out, in this connection, that the compounds of safranine and proteolytic enzymes are still proteolytically active and that methylene violet which has but little toxic action on the oxidations of *B. coli* (under the conditions employed, see Table I) will stain a smear of *B. coli* as well as some of the dyestuffs which are powerfully toxic.

The most important fact which emerges from a study of the results embodied in Table I is that all the highly toxic dyestuffs are basic in character, whilst the acid dyestuffs have comparatively little or no action at the concentrations and p_H used. This leads to the conclusion that the dehydrogenases responsible for the activation of glucose, *etc.*, are essentially acidic in character and are able to adsorb or combine with the basic and not with the acidic dyestuffs under the experimental conditions employed.

This conclusion explains the fact that dehydrogenases, in presence of their substrates, do not reduce dyestuffs in the order expected from their oxidation-reduction potentials. It is clear that the rate of reduction is dependent not only on the reduction potential of the dye but on its access to, *i.e.* its adsorption or combination with, the centre responsible for the activation of the substrate. The basic dyestuffs are more accessible, the acidic less

so, and it is thus found that the sulphonated (acidic) dyes are reduced at a much smaller speed than would be anticipated from the magnitude of their reduction potentials [Quastel and Wooldridge, 1927].

It is of considerable interest that Bismarck brown, which is a basic dye, has but little toxic action on the oxidations of *B. coli*. It is therefore apparent that although basicity of a dyestuff plays an important part in determining the combination of dyestuff and dehydrogenase, the basicity is not by any means the only factor involved; the structure of the dyestuff molecule is also concerned. It is presumably this fact which explains the relatively different actions of the dyestuffs on the oxidations of succinate and lactate. The importance of even slight differences in the structures of the dyestuffs is seen in the effects of safranine and methylene violet. The former is highly toxic to the oxidations of *B. coli*, the latter negligibly so.

It is evident from these results that it is of the highest importance, in using dyestuffs, *etc.*, for the measurement of r_H of cells, to have some knowledge of their effects on the oxidative mechanisms of the cells. Entirely fictitious results will be obtained by the use of dyestuffs having selective and toxic actions.

It is of interest that cyanin chloride[1], a natural anthocyanin, has but little action on the oxidative mechanisms of *B. coli*. It would be expected *a priori* that the natural colouring matters would not have a poisoning action on the oxidative mechanism of the cell.

Phosphates and the toxic action of dyestuffs.

The results given above all relate to suspensions of *B. coli* exposed to a 1/5000 aqueous solution of dyestuff in presence of phosphate buffer at p_H 7·4. It is found when using the organism at various concentrations and of varying activity, that (quantitatively) variable results are obtained. This is particularly the case with methylene blue, with which, in examining the effect of the dyestuff on the oxidation of succinate by *B. coli*, an inhibition of 70 % has been sometimes observed and at other times of only 30 %. The reason is doubtless partly connected with the state of the organism, but it is connected to a far greater degree with the nature of the constituents present in the solution in which the organism is suspended.

In the course of an investigation on the action of methylene blue on the rate of succinate oxidation by *B. coli*, it was found that the presence of phosphate had a most marked influence on the toxicity of the dyestuff. To study the rate of oxidation in the absence of phosphate, use was made of veronal buffer, p_H 7·4, made up as suggested by Michaelis [1930]. It was shown in the first place that the replacement of phosphate buffer by veronal buffer of the same p_H (7·4) had little or no effect on the rate of oxidation of succinate by normal *B. coli*. If, however, the *B. coli* were exposed to methylene blue (1/5000) in presence of veronal buffer, p_H 7·4, for 30 minutes at 37°

[1] We are indebted to Miss Rose Scott-Moncrieff for a specimen of this substance.

previous to the addition of the succinate, the oxidation of the latter was inhibited by over 90 %; whereas exposure to the dyestuff under the same conditions but in the presence of phosphate buffer, p_H 7·4, resulted in an inhibition of about 30 %. The following figures illustrate this:

O_2 uptake in 1½ hours in mm.³ at 37° (in presence of 0·067 *M* succinate) by:

1 cc. *B. coli* in presence of phosphate buffer, p_H 7·4	665
1 cc. *B. coli* in presence of veronal buffer, p_H 7·4	625
1 cc. *B. coli*, previously treated with 1/5000 methylene blue in presence of phosphate buffer, p_H 7·4	475
1 cc. *B. coli*, previously treated with 1/5000 methylene blue in presence of veronal buffer p_H 7·4	29

It was then shown that the toxic effect of methylene blue on *B. coli*-succinate oxidation in presence of veronal buffer could be partly reversed by the subsequent addition of phosphate buffer at the same p_H. This is indicated by the following figures:

Oxygen uptake in 2 hours in mm.³ by:

(*a*) 1 cc. *B. coli* exposed to 1/5000 methylene blue solution in presence of phosphate buffer *M*/25, p_H 7·4, for 30 minutes, succinate alone then being added	505
(*b*) 1 cc. *B. coli* exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7·4, for 30 minutes, succinate alone then being added	25
(*c*) 1 cc. *B. coli* exposed to 1/5000 methylene blue solution in presence of phosphate buffer *M*/25, p_H 7·4, for 30 minutes, a mixture of succinate and veronal buffer, p_H 7·4, then being added	538
(*d*) 1 cc. *B. coli* exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7·4, for 30 minutes, a mixture of succinate and phosphate buffer, p_H 7·4, then being added	255

The fact that there is a recovery in oxygen uptake when phosphates are added subsequently to the exposure of the organism to the dyestuff in presence of veronal buffer, indicates that the phosphates must be able to remove methylene blue to some extent from combination with the enzyme. This seems the only feasible explanation, for the fact that replacement of phosphate buffer by veronal buffer does not result in a diminished rate of oxidation of succinate by the untreated organism shows that phosphates are not essential to the oxidation of succinate. Moreover veronal cannot have the effect of making methylene blue more toxic, for the addition of veronal to the organism exposed to the dye in presence of phosphate buffer does not result in a diminished rate of oxidation of succinate. There is the possibility that phosphates and methylene blue compete for the same groups in the enzyme but there is no evidence as yet for this.

Other basic dyestuffs such as toluidine blue and malachite green behave in a similar manner to methylene blue. The acid dyestuffs, however, *e.g.* water blue and Congo red, which in phosphate buffer at p_H 7·4 have little or no toxic action on succinate oxidation by *B. coli* have just as little action in veronal buffer at the same p_H.

It seems likely that the action of the phosphate is to combine with the basic dyestuff ions and prevent their combination with the acid groups of the enzyme, or if such combination has been effected, to accomplish a reversal,

by what is tantamount to an eluting action on the adsorbed or combined dyestuff.

If for veronal buffer, p_H 7·4, there be substituted $M/20$ glycine solution brought to p_H 7·4 by the addition of a little alkali, the toxic action of methylene blue on *B. coli* (towards succinate oxidation) is greatly reduced, the oxygen uptake being nine times that which is obtained after exposing the organism to the dyestuff in veronal buffer and about half that which occurs after exposing the organism to the dyestuff in phosphate buffer. Glycine has a protective action similar to, but much less than, that of phosphate at p_H 7·4. Clearly the magnitude of the protective action of the buffer on the organism will be dependent not only on the concentration of buffer ions but on the relative affinities of the buffer ions and the acid groups of the enzyme for the dyestuff. In this connection the work of Hirschfelder and Wright [1930] and of Stearn and Stearn [1930] on bacteriostasis and the equilibria between dyestuff and proteins is of importance.

Oxidations by muscle and brain.

Meyerhof [1917, 1918] showed that the respiration of micro-organisms (staphylococci) was inhibited 10 to 40 % by methylene blue but that the respiration of the same organisms, after exposure to acetone, increased in the presence of methylene blue. Fleisch [1924] demonstrated that the addition of methylene blue to muscle tissue in presence of HCN brought about an increased rate of oxidation of succinate. Barron [1929] found that the addition of methylene blue to living cells (sea urchin, star fish eggs) and red blood cells produced an increased oxygen consumption. It is clear that the dyestuff is able to play a catalytic role in oxidations on account of its reversible nature and the fact that its leuco-derivative can be oxidised by molecular oxygen without the intervention of a dehydrogenase system. The catalytic action of dyestuffs, generally, on the oxygen consumption of living cells is dependent, as shown by Barron and Hoffman [1930], on their reduction potentials and their ability to penetrate into the cell. The results given in Table I show that the acid or basic nature of the dyestuff and its access to (or adsorption or combination with) the centre at which the hydrogen donator is activated influence considerably its effect on oxidations. In these experiments the organism was exposed to the dyestuff for a certain period before the addition of the substrate under investigation.

It has been a usual practice, in investigations on dyestuffs, to add the substrate (*e.g.* glucose) at the same time as the dyestuff to the suspension of cells. If the oxidation of the substrate, under these circumstances, is rapid, there may be insufficient time for the dyestuff to exert a marked toxic action on the enzymes involved and the observed rate of oxidation will not be complicated by this factor. This, however, is only an ideal condition, for circumstances are usually such that the rate of reduction of the dyestuff is not

extremely rapid. The nature of the buffering substances, too, will have a decided influence on the rates of oxidation in presence of the toxic dyestuffs.

When 0·5 g. fresh muscle is exposed to dyestuff prior to the addition of succinate, the experiment being carried out in precisely the same way as has been described for the bacteria, the toxic action of such basic dyestuffs as malachite green and methyl violet becomes apparent. Illustrative results are shown in Table II.

In a typical experiment 0·5 g. freshly minced rabbit muscle was placed in each vessel of the Barcroft apparatus, and phosphate buffer, p_H 7·4 (to give a final concentration of $M/15$), and dyestuff (to give a final concentration of 1/5000) added. The apparatus was allowed to shake at 37° for 30 minutes, and then succinate (to give a final concentration of 0·067 M) was added to the right-hand vessel and saline to the left-hand vessel. The rate of oxygen uptake was measured and compared with the control where the muscle was not exposed to dyestuff. It is important in these experiments to have in the control vessels a volume of distilled water equal to that of the dyestuff solution which is placed in the experimental vessels. If normal saline be used instead of distilled water, confusing results are obtained owing to differences in osmotic tension.

In one experiment, for instance, the oxygen uptake by 0·5 g. muscle, unexposed to dyestuff, in presence of succinate was 386 mm.^{3} in 2 hours; the oxygen uptake by the muscle, exposed to 1/5000 malachite green, in presence of the succinate was 150 mm.^{3} in 2 hours. When acid fuchsine and Congo red were substituted for malachite green, the oxygen uptakes in 2 hours were 355 and 375 mm.^{3} respectively.

Muscle tissue deteriorates, on standing at 0°, at a far greater rate than a suspension of bacteria, so far as the oxidation of succinate is concerned, and this factor is of importance in estimating the effects of dyestuffs. The results with muscle cannot be compared quantitatively with those found with bacteria, for there is a great difference in the quantity of biological material used. Qualitatively, however, there appears to be a parallel between the action of dyestuffs on bacteria and on muscle.

Methylene blue and toluidine blue must be considered separately, for on certain occasions both these dyestuffs have given rise to an increased rate of oxidation of succinate and on others to a decided inhibition. Much depends on the state of the muscle at the time of experiment, and it seems clear that the variability in results is due to the interplay of two factors, (*a*) a toxic action on the enzymes involved in the oxidation of succinate, leading to inhibition of the rate of oxidation, (*b*) a catalytic action, due to reduction of the dyestuff and subsequent oxidation, leading to an increased rate of oxidation. If these two effects happen to balance each other, the net effect of the addition of methylene blue is zero—this has occurred on several occasions. On the other hand, on two occasions accelerations of 42 and 46 % have been noted and on another an inhibition of 31 % was found. Much the same has been found with toluidine blue.

If instead of fresh muscle, well-washed muscle be used, methylene blue has a slightly more vigorous toxic action. If, however, using washed muscle, phosphate buffer be replaced by veronal buffer at the same p_H, methylene blue has a very decided toxic action—an effect precisely similar to that found with the bacteria. Thus in one experiment, using well-washed rabbit muscle, methylene blue brought about a 6 % inhibition in presence of phosphate buffer ($M/15$, p_H 7·4) and a 60 % inhibition in presence of veronal buffer (p_H 7·4).

Brain tissue (sheep's whole brain has been chiefly studied) appears to be almost unaffected by the dyestuffs. In no case (see Table II) has an inhibition

Table II. *Percentage inhibition by dyestuffs of succinate oxidation by muscle and brain*

Dyestuff (1/5000)	Muscle	Brain
Methyl violet	30	0
Malachite green	50	0
Gentian violet	15	0
Methylene blue	0	0
Toluidine blue	0	0
Acid fuchsine	0	0
Water blue	4	0
Congo red	3	0

of the oxidation of succinate, due to the exposure of the tissue to dyes, been observed. Slight accelerations, *e.g.* with methyl violet, malachite green, acid fuchsine, toluidine blue, have been noted but these are irregular. For example:

The O_2 uptake in 1½ hours in presence of phosphate buffer, p_H 7·4, and succinate in mm.³

(*a*) by 0·5 g. sheep's brain 514
(*b*) by 0·5 g. sheep's brain exposed to 1/5000 methyl violet 560
(*c*) by 0·5 g. sheep's brain exposed to 1/5000 toluidine blue 571
(*d*) by 0·5 g. sheep's brain exposed by 1/5000 malachite green 553

The difference between the effects of, say, methyl violet or malachite green, on the oxidations of brain and muscle tissue is very striking. It seems unlikely that the enzymes involved in succinate oxidation in brain are so differently constituted from those in muscle or bacteria that they are not attacked by the basic dyestuffs—it is more reasonable to suppose that the enzymes are protected either by an inability of the dyestuffs to reach the site of the enzymes in the cell or because there are constituents present in the tissue which combine preferentially with them.

Clearly, the locality of the enzymes in the tissues and the nature of their immediate environment are most important factors in determining the toxicity of dyestuffs.

Summary.

1. The action of 29 dyestuffs on the oxidations of glucose, lactate, succinate and formate by *B. coli* has been investigated.

2. It is shown (*a*) that all the dyestuffs which have a vigorous toxic action are basic in character, whilst the acid dyestuffs have little or no action; (*b*) that the molecular structure of the dyestuff plays an important part in

determining toxicity; (*c*) that specificity of behaviour towards the dehydrogenases is exhibited.

3. The toxicity of the basic dyestuffs is greatly dependent on the nature of the buffering agent present.

4. The action of dyestuffs on the oxidation of succinate by muscle tissue is similar to that found with bacteria. In the case of methylene blue or toluidine blue two effects take place, (*a*) a toxic action leading to inhibition, (*b*) a catalytic action leading to an increased rate of oxidation, due to the reversible nature of the dye. As in the case of bacteria, the presence or absence of phosphate influences considerably the toxicity of the dyestuff.

5. The dyestuffs, so far examined, appear to have no inhibitory action on the oxidation of succinate by brain tissue.

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LXXIII. THE EFFECT OF ADDING VITAMIN A TO A RACHITOGENIC DIET.

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It is generally held that a direct relation exists between growth-rate and intensity of rachitic lesions in the human subject. Thus Hess [1929] writes, "In general, it may be stated that the more rapid the growth, the greater the tendency to the development of rickets....Animal experiments confirm this general point of view....All investigators who have used the rat as the experimental animal have reported similar experiences." There are abundant references in the literature to "spontaneous healing," and this has in very many cases been associated with a diminished food intake and a retarded rate of growth.

Recently Coward and Cambden [1929] made a special investigation of this matter. They made use of a modified McCollum line test, and did not find any significant correlation between intensity of rickets and failure to grow. Their results were in contradiction to those reported by Bills, Honeywell and MacNair [1928], who remarked "It is imperative to discard all rats which lose even as little as 1 g." Bills also used the line test technique; whereas the curative period in Coward's experiments was 10 days, that in Bills's investigations was only 5. The experiments of Coward, of Bills and of other investigators who have reported results in either sense, were not primarily designed to investigate the particular point under discussion; their conclusions have been drawn from a considerable body of experimental evidence bearing mainly on different points. Bills has recently re-affirmed the view expressed in the earlier paper [Bills, Honeywell, Wirrick and Nussmeier, 1931], and has repeated the exact words of the sentence already quoted.

The following investigation was planned and concluded before the publication of the paper last mentioned. It was designed with the specific object of investigating directly the relationship between increase in growth and intensity of rickets.

The matter is of considerable importance in the assay of vitamin D. The rachitogenic diet most used in this country is Steenbock's 2965. In this the only source of vitamin A is yellow maize. It is known that maize varies in its vitamin A content, the white-cap variety being practically devoid of it [*e.g.* Steenbock and Coward, 1927; Hauge and Trost, 1928]. There is even no reason for assuming that all samples of yellow-cap maize are equally potent

sources of vitamin A. It might therefore happen that a particular batch of diet 2965 was actually deficient in the growth promoting properties associated with vitamin A (or carotene). If this batch were being used in the comparison of a sample of cod-liver oil with irradiated ergosterol, the presence of vitamin A in the former might bring about markedly greater growth in the animals receiving it than in those receiving the irradiated ergosterol. If this increased growth were associated with greater intensity of rickets, the comparison of antirachitic potency would be thereby completely invalidated. Accordingly the investigation here described was undertaken to determine whether the daily addition of fresh carrot to diet 2965 affected the degree of rickets produced by the diet (*a*) when no source of vitamin D was added and (*b*) in the presence of two different levels of vitamin D supplement.

Thirty-one animals were divided into three groups, the first receiving no source of vitamin D, the second a very small daily dose, in one drop of olive oil, and the third eight times as much as the second, dissolved in the same weight of the same olive oil. The doses administered in the two cases were approximately 0·1 and 0·8 Medical Research Council units daily; no great stress is laid upon the absolute amount of vitamin D administered, but it is definitely known that the two dosages were in the ratio of 1 : 8, since they were made up from the same preparation of irradiated ergosterol. Each group was divided into two sub-groups, in one of which each animal received 1 g. of fresh carrot daily. Although no assay of vitamin A (or carotene) was carried out on the samples of carrot used, similar samples purchased at the same time were found to be ample in daily doses of 1 g. to restore growth to animals "run out" on a vitamin A-free diet. In view of the unanimity of the literature on the subject, it can safely be assumed that the animals receiving the supplementary doses of carrot were receiving adequate supplies of vitamin A.

The experimental period was 5 weeks; the animals were all kept in separate cages. One hind-leg and one fore-leg of each animal were taken for extraction and ashing, and photographic records were kept of the "lines" from the other tibia of each animal. In general the appearance of the lines was quite consistent with the figures obtained by chemical analyses. The criterion of rickets used in these experiments has been the percentage of ash in the moisture- and fat-free bones, [A]. This figure is closely related to the "rachitic index," used by Chick and Roscoe [1926], which is the ratio of the ash to the non-fatty organic matter in the bone. This last figure, denoted R, is related to [A] and to R.I. (the rachitic index) in the following way:

$$\text{R.I.} = \frac{[A]}{R} = \frac{[A]}{100 - [A]}$$

When the value of [A] is 50, the R.I. is clearly 1.

Records were kept of the initial and final weights of all the experimental animals. As far as possible animals of the same sex and from the same litter (for which it is proposed to use the term isogenic animals) were distributed over the various dosages administered.

The actual increases in weight appeared to bear no relation to the weights of the animals at the beginning of the experimental period, but rather, as was to be expected, to show some correlation with the individual litters. Since the intensely inbred London strain of Wistar rat was used throughout, the differences can hardly have been genetic, and are probably associated with differences of prenatal nutrition and fat-soluble vitamin storage.

The value for [A] was obtained on the bones after they had been extracted with alcohol to constant weight. We have found this to be an essential precaution, since the size and porosity of different bones are the main factors affecting the ease with which the fat and moisture can be removed from them. We have also found that subsequent ether extraction is unnecessary since it never causes a further loss in weight of more than 1 or 2 mg. The bones were ashed in a muffle at dull red heat, and were also weighed a second time after a second heating, although the latter never produced any significant change in weight.

Table I shows the distribution of litters, animals, and doses, the value found for [A] and for the increase in growth during the experimental period, δM (in brackets).

Table I. *Values for* [A] *and* δM *(in brackets) for 31 animals from 3 litters.*

			Vitamin D			
		No vitamin D	0·10 unit		0·80 unit	
Litter		No carrot	Carrot	No carrot	Carrot	No carrot	Carrot
1534	7 ♂	40·9 (48)	45·5 (62)	42·3 (60) 37·0 (47)	39·8 (52)	44·4 (59)	46·5 (45)
	7 ♀	34·2 (41)	34·2 (33)	34·8 (42)	38·7 (44) 46·4 (32)	40·1 (47)	45·1 (43)
1529	7 ♀	33·5 (22)	32·8 (31) 32·3 (38)	40·1 (38)	42·3 (40)	41·5 (38)	44·2 (35)
1386	6 ♂	38·2 (74)	36·4 (72)	38·8 (78)	28·4 (63)	41·6 (80)	48·4 (86)
	4 ♀	33·4 (44)	31·7 (51)	39·1 (58)	—	36·1 (45)	—
Averages		36·0 (46)	35·5 (48)	38·7 (54)	38·7 (46)	40·7 (54)	46·1 (52)
		35·8 (47)		38·7 (50)		43·4 (53)	

Discussion of results.

It appears that the batch of diet 2965 used in these tests was an adequate source of vitamin A, under the conditions of the experiment, since a daily supplement of 1 g. of carrot did not appear to bring about a significant change in growth-rate. The average increases for all animals during the whole 5 weeks were:

without carrot	(7♂, 9♀)	51
with carrot	(6♂, 9♀)	48

The difference between the averages is not more than could be accounted for by the slightly different sex-ratio and other normal variations and is certainly not significant.

It also appears that there is no correlation whatever, under the conditions and within the limits of this experiment, between growth-rate and severity of rickets, as chemically assayed and corroborated by the line test technique. Fig. 1 shows [A] plotted against δM for all the animals.

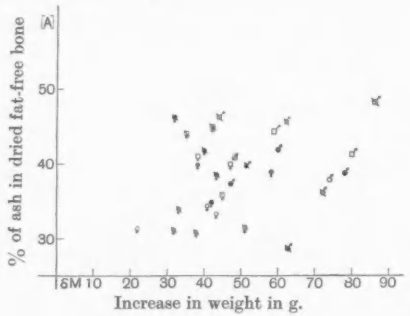

Fig. 1.

Without carrot.	With carrot.
○ without vitamin D.	◔ without vitamin D.
● with 0·1 unit vitamin D.	◕ with 0·1 unit vitamin D.
□ with 0·8 „ „	◔ with 0·8 „ „

The carrot appears to have exercised no antirachitic effect when given unsupplemented at a daily level of 1 g. There is a suggestion that higher, and nearly prophylactic, doses of vitamin D are made more effective in the presence of increased vitamin A (or carotene), but the number of animals used was insufficient, and the differences in bone-ash percentage too fluctuating, for this to be anything more than an indication. Nevertheless, all four relevant pairs of isogenic animals show this effect, and its significance seems probable. Steenbock *et al.* [1930], amongst others, have called attention to the absence of evidence that "the various factors operative in bone production act independently and consistently without one modifying the influence of the other."

Summary.

1. Addition of 1 g. of fresh carrot daily to the diet of animals on Steenbock's diet 2965 did not significantly affect growth-rate, whether in the absence or presence of vitamin D.

2. No evidence was found, under the conditions of the experiment, for any correlation between severity of rickets and rate of growth.

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LXXIV. THE INFLUENCE OF CALCIUM ON THE RATE OF DIFFUSION OF SUGARS THROUGH SURVIVING INTESTINE.

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(*Received March 9th, 1931.*)

In previous work on this subject [Macleod *et al.* 1930; Auchinachie *et al.* 1930], the fluid bathing the surviving segments of intestine was always sugar-free Tyrode's solution. In view of the well-known effects of electrolytes on the physiological activity of living cells, it was decided to determine whether the selective properties of the intestinal epithelial cells would be affected by varying the composition of the outer fluid.

The procedure was the same as in previous experiments except that, as it was necessary to prepare the segments in sugar-free but otherwise normal Tyrode (called "normal Tyrode" in text), each segment was thoroughly washed in Tyrode of the composition to be used for the test, so as to ensure that all traces of fluid, except that whose influence was to be studied, would be removed from the intestine. Rabbit's small intestine was used, and its generally greater permeability in an aboral direction was ruled out by suitable selection of segments for control experiments. The volume of outer fluid was 50 cc., and 4 cc. 0·75 *M* sugar solution were generally placed in the lumen.

Results.

When the rates of diffusion of 0·75 *M* glucose through the walls of segments bathed in normal and in Ca-free Tyrode respectively were compared, the rate was found to be greater in the former case, as the following figures from a typical experiment show:

Sol.	45	90	135 mins.
Ca-free Tyrode	29	103	185 } mg. glucose per
Normal Tyrode	49	137	275 } 100 cc. fluid

The segments bathed in Ca-free Tyrode showed scarcely any movements; whereas those bathed in normal Tyrode contracted vigorously and undoubtedly increased the pressure of the lumen contents. This factor was not, however, responsible for the more rapid diffusion, because inhibition of the movements by continuous slow addition of a weak adrenaline solution did not alter the nature of the result. By starting with all the segments immersed in Ca-free Tyrode and then adding the usual amount of $CaCl_2$ to one series of beakers,

after a suitable interval, the accelerating effect of the $CaCl_2$ on diffusion was always elicited (see Fig. 1). When the diffusion was allowed to proceed into

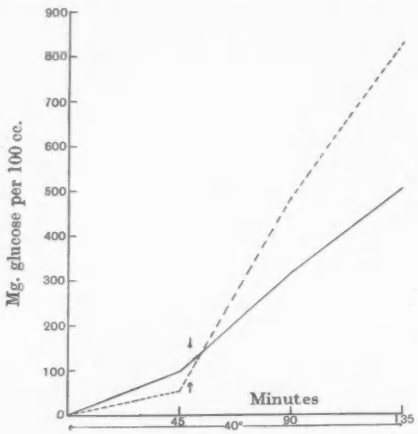

Fig. 1.

——— Ca in outer fluid. Oxalate added at ↓.
– – – – – No Ca in outer fluid. Ca added at ↑.

normal Tyrode for a period and the Ca then precipitated by the addition of an equivalent amount of oxalate to one beaker a slowing of the rate of diffusion occurred (see Fig. 1). Similar results were obtained by adding an equivalent amount of citrate instead of oxalate. This result shows that the accelerating effect of calcium on the diffusion of glucose is due to calcium ions and not to un-ionised calcium, since the citrate, while it removes Ca^{++}, leaves un-ionised calcium in solution.

We are not aware of any reason why Ca^{++} should increase the permeability of the intestinal wall. Indeed, a contrary effect would not have been surprising in view of the discussion of the literature in an earlier paper [Magee and Reid, 1927]. The foregoing results having suggested that Ca^{++} was necessary for the physiological phenomena manifested by surviving intestine, it became all the more necessary to perform experiments testing this conception more stringently.

Effect of temperature. Adjacent segments containing the same volume of 0·75 *M* glucose were suspended in Ca-free and in normal Tyrode respectively and kept at 0° for a period. After withdrawing samples from the outer fluid the temperature was suddenly raised to 40°. Fig. 2 shows a typical result. It will be seen that after 30 minutes at 0° the amounts of glucose that diffused out were approximately equal, whereas at 40° the rate of diffusion was much more rapid in the case of the segment bathed in normal Tyrode. Since all physiological activity was in abeyance at 0° the equality in the rates of diffusion may be taken as proof that Ca^{++} would not affect the diffusion of glucose, either on

account of attraction of sugar by Ca^{++} in the outer fluid, or through an action on the texture of the gut wall. The more rapid diffusion in the case of the segment bathed in normal Tyrode at 40°, therefore, indicates that the physiological activity evoked by the rise in temperature was only possible in the

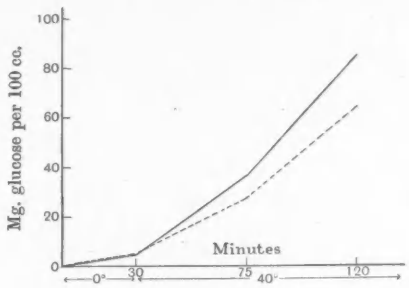

Fig. 2.

——— Ca in outer fluid. – – – – – No Ca in outer fluid.

presence of Ca^{++}. The results given below show that it made little difference to the rate of diffusion whether the Ca^{++} were present in the inner or outer fluid.

Inner fluid	Outer fluid	30	60	90 mins.	
4 cc. 0·75 *M* glucose	Ca-free Tyrode	17·1	43·4	80·5	mg. glucose per 100 cc. outer fluid
4 cc. 0·75 *M* glucose in 0·02 % $CaCl_2$	Ca-free Tyrode	21·0	50·3	101·2	
4 cc. 0·75 *M* glucose	Normal Tyrode	16·6	50·9	107·0	

Comparison of glucose and xylose. Previous work showed that, when equimolecular solutions of glucose and xylose were placed in adjacent loops, glucose generally passed into the outer (normal) Tyrode more rapidly than xylose. On repeating these experiments with Ca-free Tyrode no evidence of selective uptake of glucose could be demonstrated, but instead xylose passed out more rapidly than glucose, as was found in the case of dead intestine. This result again shows that the surviving intestine requires Ca^{++} to enable it to discriminate between the physiological sugar, glucose, and the foreign sugar, xylose.

Different concentrations of sugar. When equal volumes of *M* and 0·75 *M* glucose were placed in adjacent loops of surviving intestine, suspended in Ca-free Tyrode, diffusion was more rapid in the case of the *M* solution. This result, which is in accordance with the diffusion laws, is contrary to those previously obtained for diffusion into normal Tyrode. In this case diffusion was maximal with 0·75 *M* solutions.

It will, therefore, be seen that when the surviving intestine is examined by the above-described methods it fails to display in the absence of Ca^{++} those selective properties which are shown in the presence of Ca^{++}. Further experiments suggest, however, that Ca^{++} is not the only ion whose presence is

necessary for the manifestation of this selective action. Thus, when the diffusion of glucose was compared with segments bathed in normal and K-free Tyrode, the rate was greater in the former case.

Sol.	30	60	90 mins.
Normal Tyrode	39	91	154 } mg. glucose per
K-free Tyrode	38	87	139 } 100 cc. outer fluid

Similarly, diffusion was more rapid when the outer fluid was normal Tyrode of p_H 7·4–7·6 than when this was of p_H 5·4. The promotion of diffusion by the higher p_H was also shown to be independent of the concentration of HCO_3^-. But further work will be necessary before the rôles played by these ions in absorption can be clearly stated.

This investigation as a whole also shows that the passage of glucose through the wall of surviving intestine into an outer environment of Tyrode's solution is, to some extent, of the nature of an active transport of sugar molecules by the activity of the epithelium as well as a process of simple diffusion. But, as stated in an earlier paper, the effect of the surviving activity of the cells is liable under severe test to be masked by the purely physical phenomena.

Summary.

In experiments with surviving rabbit's intestine it has been found that glucose diffuses more slowly from the lumen into Ca-free than into Ca-containing outer fluids, the process in the former case being a physical one but in the latter partly of the nature of a selective action.

The presence of calcium ions in the environment is indispensable for the manifestation of selective action of the intestinal wall towards sugars placed in the lumen.

We desire to express our thanks to Professor Macleod for advice and criticism.

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LXXV. ON THE INFLUENCE OF SERUM ON ENZYMES, WITH SPECIAL REFERENCE TO ITS ACTION ON TRYPSIN.

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(*Received January 21st, 1931.*)

Introduction.

The influence of serum on enzymes may be considered kinetically as falling into one of two categories. Either the enzyme is influenced by the presence of a similar enzyme in serum, in which case an apparent acceleration is produced, or else the action is associated with an absence from serum of the enzyme in question, in which case the action is one of true acceleration, or inhibition, as the case may be; the mere fact that serum accelerates or inhibits an enzyme constitutes in itself incomplete evidence of the nature of the action. In a recent publication [1930], I have shown how failure to recognise this principle has already vitiated work done on the subject.

It is usually a simple matter to determine whether an apparent acceleration is due to the presence of the enzyme in serum, or whether the acceleration is a true one; greater discrimination however is necessary to establish the presence of both factors if present simultaneously in a case of acceleration, and to determine to what extent each is responsible for the effect observed.

Serum-enzymes. In some instances the action of serum is wholly explained by the presence of a serum-enzyme, as in the case of serum-diastase; in other cases the action is only partially explained by the presence of an enzyme in serum, an example of which is human serum acting on lipase.

The enzymes recorded in serum include protease, peptidase, diastase, lipase, catalase, invertase, and maltase. It is important to recognise that the distribution of enzymes in serum is by no means identical in different species.

Thus, while diastase is fairly widespread, occurring in man, sheep, cow, rat, and guinea-pig, it is absent from rabbit [Luers and Albrecht, 1926]. Lipase I have also found in man, sheep, cow, rat and guinea-pig. Maltase was not found in guinea-pig serum [Blacklock, Gordon and Fine, 1930] but has been recorded in pig serum. Invertase I have failed to find in a number of animals, including man, but under experimental conditions (*e.g.* following intraperitoneal injections of a commercial preparation of invertase) I have

found it in guinea-pig serum, where it persisted for over 30 hours in one instance [Fine, 1930].

The influence of serum on enzymes not ascribable to the presence of serum enzyme. It is this category of the influence of serum on enzymes that is the subject of the present paper.

Even when serum does contain an enzyme, its action may be greater than is to be accounted for by the presence of the enzyme; for example, human serum will increase the activity of lipase to a degree exceeding what would be expected from the mere addition of a lipase of the strength of serum-lipase. The total acceleration is, in this case, partly due to the presence of serum-lipase (apparent acceleration), and is partly a true acceleration.

It has been customary to explain the effect of acceleration by serum as due to "an accelerator," and the effect of inhibition as due to an "inhibitor," or, more commonly, an "anti-enzyme." I have shown however [1930] that in the case of invertase the influence exerted by serum is entirely a function of p_H, the serum acting as an accelerator over certain ranges of p_H and as an inhibitor over others.

In the following pages I have brought together evidence both from my own experiments and from the work of others, which justifies the conclusion that with most other enzymes also true acceleration or inhibition by serum is not due to specific accelerators or anti-enzymes, but to various physical conditions such as p_H or the adsorption of the enzyme by serum, the latter being particularly significant in the case of the antitryptic action of serum.

In the previous publication referred to [1930] I have already dealt with the enzyme-accelerating properties of serum, and there pointed out that serum has been known to accelerate only two enzymes—invertase and lipase. The same serum which accelerates invertase will also inhibit it if the p_H is suitably altered. The influence of p_H on the lipase-accelerating power of serum has not been worked out.

Anti-enzymes reported in serum.

The origin and significance of the term "anti-enzyme." The first observation of the phenomenon of enzyme-inhibition by serum was made by Hildebrandt [1893], who discovered that serum inhibited the action of emulsin. Hahn [1897] followed with the observation that serum inhibited trypsin, and Morgenroth [1899] found that it inhibited rennin.

Although none of these workers isolated any substances from serum which might account for the inhibition observed, they all explained the phenomenon observed by the presence of corresponding anti-enzymes in normal serum. Very early, however, Landsteiner [1900] showed, and Cathcart [1904] and others confirmed, that trypsin inhibition was a property of the albumin fraction of serum rather than of the globulin fraction, whereas it was well established that the immunological antibodies resided in the globulin fraction.

Furthermore no one could demonstrate a material increase of the enzyme-inhibiting action of serum following enzyme injections. The consensus of opinion appears to be that trypsin is the only enzyme that can evoke the response of an increased enzyme-inhibition in serum, and even so, the increase never exceeds three or four times the original amount of inhibition. Such response is to be sharply differentiated from the effects of toxin injection on antibody formation in serum: true antibodies can invariably develop to the amount of thousands of times the original antibody content.

Weil [1910] pointed out that while serum can inhibit the haemolytic action of saponin, it does not necessarily follow that serum contains an antisaponin, and, indeed, as Bayliss [1919] says, if a substance which inhibits an enzyme is entitled to be called an anti-enzyme, then sodium hydroxide is antipepsin.

The action of serum on enzymes is largely a function of p_H. The earlier workers, as Wells [1925] has pointed out, seem to have quite ignored the effect of p_H. Bayliss [1912] in examining the evidence for Hildebrandt's anti-emulsin found that the mere addition of serum to an emulsin mixture sufficiently altered the p_H to produce a corresponding fall in activity of the emulsin. Bensley and Harvey [1912] showed that the absence of free HCl from the cells of the gastric mucosa formed the best protection against the secreted pepsin: in other words, the p_H of the cell substance was in itself an adequate "antipepsin." Dragsted and Vaughan [1924] sewed the spleen and kidney into openings made in the stomach wall, and found that they were unaffected by the consequent exposure to pepsin, provided the blood supply were kept intact. In their view the mechanism of this immunity was simply the buffer action of the blood and the absorption of the HCl and pepsin into the blood-stream. Thaysen [1915] found that "antirennet" could also be accounted for by the change in p_H when serum is added to a rennet mixture: he also found that adsorption of rennet by the serum-proteins played an additional part. The previously mentioned observations of the present author [1930] on invertase afford strong evidence in the same direction.

In the case of other enzymes however the influence of serum under different conditions of p_H has not hitherto been worked out, and although p_H regulation must play some part, it is probable that a number of factors are involved in the defence of the organism against the action of excessive amounts of enzyme. Excretion is one of the chief factors, as in the case of amylase; destruction in the body probably occurs in the case of invertase [Fine, 1930]; adsorption by tissue- or serum-proteins in the case of trypsin.

At various times serum has been reported to possess an inhibitory action towards trypsin, pepsin, rennin, papain, "autolytic enzymes," lipase, amylase, invertase, laccase, catalase, tyrosinase, emulsin, urcase and fibrin-ferment. Consideration of the p_H optima of these enzymes shows however that the mere addition of serum which has a p_H of 7·45 would greatly reduce the activity of unbuffered solutions of pepsin, rennin, papain, autolytic enzymes, emulsin, and invertase. Even invertase, which is accelerated by serum when buffered

at its optimum p_H, is totally inhibited when serum is added to its unbuffered solution.

In the case of trypsin however, the p_H factor does not appear to play any part, the inhibition of trypsin by serum being, as will be shown later, purely a manifestation of the power of serum to adsorb the enzyme. Occupying an intermediate position between pepsin on the one hand, and trypsin on the other, there is a group of enzymes, of which rennin is an example, whose inhibition by serum is partly a p_H effect and partly an adsorption effect [Thaysen, 1915].

Recovery of trypsin from inhibition by serum.

Following Hahn's observation [1897] that serum contained antitrypsin, *i.e.* retarded the action of trypsin, many substances of organic nature were examined for their influence on trypsin, in the hope of some light being thrown on the phenomenon. Among these so-called inhibitors may be mentioned (1) extract of intestinal worms [Weinland, 1903; Dastre and Stassano, 1903; Hamill, 1906; Burge, 1915]; (2) egg-white [Vernon, 1904; Sugimoto, 1913; Bayliss, 1923]; (3) bacteria [Czapek, 1903; Jobling and Petersen, 1914, 4]; (4) yeast [Buchner and Haehn, 1910]; (5) body tissues generally [Levene and Stookey, 1903]; (6) cells of gastric mucosa [Blum and Fuld, 1906; Langenskiold, 1914]; (7) mucous secretion [Langenskiold, 1907]; (8) erythrocytes [Czapek, 1903]; (9) inflammatory exudates [Opie, 1905]; (10) urine [von Schoenbom, 1910; Fujimoto, 1918]; (11) unsaturated fatty acids [Jobling and Petersen, 1914, 1]; (12) lecithin [Schwartz, 1909].

An examination of the properties of these various substances showed, however, that the mechanism of their inhibition of trypsin had little in common, and that egg-white only may be considered as acting in a similar manner to serum. Among the various properties in which these two substances differed outstandingly from other trypsin-inhibiting bodies was the temporary nature of the inhibition. This property was however shared by worm extract whose behaviour generally otherwise differentiated it clearly from serum-antitrypsin.

Experimental demonstration of recovery of trypsin.

All the methods of estimating antitrypsin hitherto employed—Gross [1908] (casein precipitation); Folin and Denis [1911] (incoagulable nitrogen); viscometric method [Feldstein and Weil, 1909–10]: electro-conductivity method [Bayliss, 1923]; refractometric method [Robertson, 1918]—are subject to the criticism that when two digesting mixtures are compared, one containing serum and the other containing an equal amount of water, but otherwise the same in composition, the amount of protein in the two tubes is not the same, and therefore the amount of digestion not strictly comparable; for it must be remembered that serum is a 6 % protein solution. The technique I have used and found highly satisfactory has been as follows.

Two tubes were prepared containing equal quantities of trypsin, buffer (p_H 8), and serum; in the first the serum had been heated at 70° for half an hour to destroy antitryptic action and in the second the serum was untreated. An aliquot portion of each tube was titrated by Sørensen's method immediately the constituents were added and mixed and a fixed number of hours later. The rise in acidity indicated the amount of tryptic action that had taken place in the two tubes containing the same concentration of trypsin and substrate. If the rise in acidity in the first tube is A, and that in the second B, then the ratio $\frac{A-B}{A}$ adequately measures the antitryptic activity.

One of the chief advantages of the Sørensen method is the additional information it gives when the free and "formol" acidities are considered separately.

Using the Sørensen method and the technique I have outlined I was able to follow the course of digestion of serum-protein by trypsin, and in this way not only obtained the Dastre [1903] effect with serum, *i.e.* enzyme recovery following preliminary inhibition, but found that almost invariably the recovery of the trypsin from inhibition was such that the inhibited trypsin ultimately showed greater activity than the trypsin acting freely on heated serum.

The experiments showing this recovery were carried out as follows:

Two tubes were incubated whose contents were as below:

	Tube 1	Tube 2
	cc.	cc.
Trypsin	0·2	0·2
Serum	0·1	0·1 (inactivated)
Buffer p_H 8	0·7	0·7

(*a*) The source of trypsin was Liquor trypsin co. (Benger's) and before use it was neutralised with $N/10$ NaOH to p_H 8 and then further diluted to 50 % of original strength.

(*b*) The buffer was prepared according to Clark and Lubs.

(*c*) The serum in tube 2 was inactivated by heating at 70° for half an hour. It was heated together with the buffer so as to avoid the difficulty of mixing afterwards; during the heating the tube was stoppered to avoid loss of volume by evaporation.

(*d*) After tube 2 was cooled, the trypsin was added to the two tubes from which, after adequate mixing, 0·1 cc. was immediately withdrawn for titration of acidity with $N/100$ NaOH. A layer of toluene was added to the tubes before incubating.

The method of titration was that employed by Wigglesworth [1928] in his investigation of cockroach protease. The pipetted fluid (0·1 cc.) was added to 5 cc. of distilled water in a test-tube; 5 drops phenolphthalein solution (0·05 % in 50 % alcohol) were added, and $N/100$ NaOH was added from a 2 cc. microburette graduated in 0·01 cc. The alkali was added until a pink colour developed identical with that of a tube (of similar make) containing

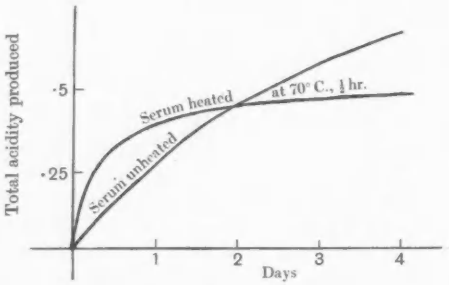

Fig. 1. Digestion of 10 % guinea-pig serum by 10 % trypsin. This shows recovery of inhibited trypsin until activity is greater than that of uninhibited trypsin.

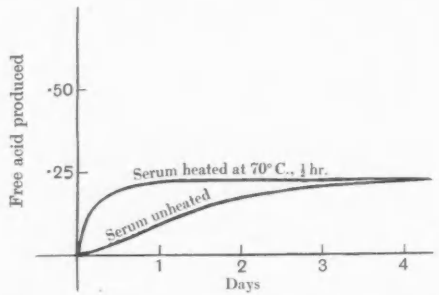

Fig. 2. Free acid produced in digestion of 10 % serum by 10 % trypsin. This shows how free acid produced by inhibited trypsin ultimately equals amount formed by uninhibited trypsin.

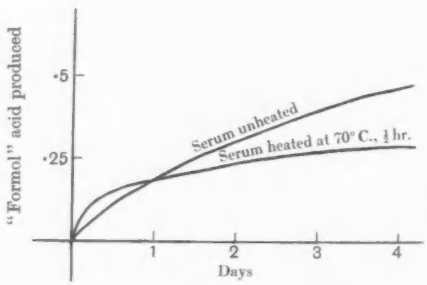

Fig. 3.

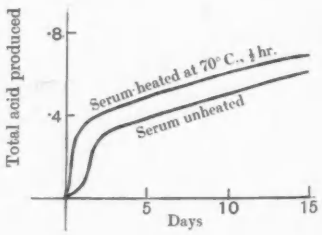

Fig. 4.

Fig. 3. "Formol" acid produced in digestion of 10 % serum by 10 % trypsin. This shows how inhibited trypsin ultimately forms more "formol" acid than uninhibited trypsin.

Fig. 4. Total acid curves of digestion of 10 % serum by 10 % trypsin. Recovery of inhibited trypsin is incomplete in this case: the serum was obtained from a guinea-pig which had recently had a large injection of trypsin.

5 cc. of buffer solution of p_H 9 *plus* 5 drops of 0·05 % phenolphthalein in 50 % alcohol. The end point was remarkably sharp.

The figure thus obtained was a measure of the "free acid" present; the "formol acid" was obtained by adding 0·5 cc. of 50 % neutralised formalin (p_H 8) and titrating further with alkali till the end point was reached again. When desired the free and "formol" acids were combined in a single figure—the "total acid."

Determinations of the acid present in 0·1 cc. of the incubated mixtures were carried out from time to time, and in this way curves of digestion were plotted.

Fig. 1 is the curve of a typical experiment, showing how the initial inhibition of trypsin is finally replaced by digestion more active than in the tube where trypsin was not initially inhibited. An analysis of Fig. 1 in the form of separate curves of free and of "formol" acid formation (Figs. 2 and 3) throws some light on the phenomenon. The latter figures show that the final superior digestion of unheated serum applies only to "formol" acid formation: on no occasion have I found more free acid ultimately formed in the presence of unheated serum (tube 1) than of heated serum (tube 2), whereas "formol" acid formation was almost invariably greater in tube 1 than in tube 2, provided digestion was sufficiently prolonged.

In some cases, even after weeks of incubation, the total acid in tube 1 remained below the value in tube 2. An example of this type of incomplete recovery is shown in Fig. 4 the curve of which was obtained from the serum of a guinea-pig which 7 days previously had received a large injection of Liquor trypsin co. An analysis of the total acid curve, in Figs. 5 and 6, shows that the incompleteness of recovery of the trypsin affects only the free acid formation, the "formol" acid curves intersecting in the same way as in Fig. 3.

Figs. 7, 8 and 9 are the total, free, and "formol" acid curves of digesting mixtures containing

10 % gelatin	0·5 cc.
Trypsin 50 %, neutral ...	0·5 cc.
Buffer p_H 8·5...	0·5 cc.
Serum...	0·2 cc.
Saline 0·88 %	0·3 cc.

Here also the greater digestion of unheated serum affects the "formol" acid curve only. The addition of the gelatin appears to hasten the recovery of the trypsin.

Interpretation of foregoing results. (*a*) It would appear on examining Fig. 1 that serum can not only retard the action of trypsin, but can at a later stage enhance it, after passing through an intermediate stage during which trypsin becomes gradually released from its bonds. In the Fig. 4 type of experiment the stage of enhancement is not reached, though the trypsin has recovered considerably from the inhibiting action of serum.

(*b*) An examination of the free and "formol" acid curves separately shows very strikingly that the transition from inhibition to enhancement applies

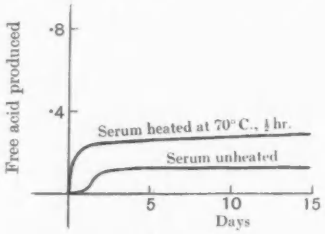

Fig. 5.

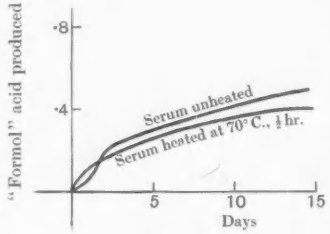

Fig. 6.

Fig. 5. Free acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, the free acid only being considered.

Fig. 6. "Formol" acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, "formol" acid only being considered.

only to the "formol" acid formation; the extent of formation of free acid with unheated serum never exceeds that with heated serum although, provided

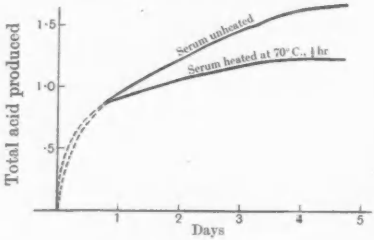

Fig. 7.

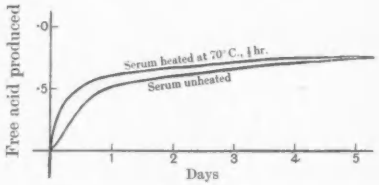

Fig. 8.

Fig. 7. This shows accelerated recovery of inhibited trypsin in presence of gelatin. The dotted part of the curves is hypothetical, as no estimation of acidity was made during that interval.

Fig. 8. Free acid curves (2·5 % gelatin present). This shows the same relation between the free acid formed by inhibited and by uninhibited trypsin as in Fig. 2.

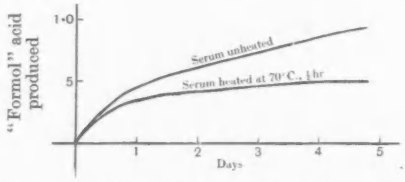

Fig. 9. "Formol" acid curve (2·5 % gelatin present). This shows the same relation between "formol" acid formed by inhibited and uninhibited trypsin as in Fig. 3.

digestion is sufficiently prolonged and the trypsin is fairly active, the same amount of free acid is finally produced in the two tubes: Fig. 5 shows a marked but not complete free acid recovery in the unheated serum tube.

(*c*) Serum therefore possesses the property, destroyed by heat, of temporarily retarding formation of free and "formol" acid by trypsin, and of subsequently accelerating "formol" acid formation only.

How is this behaviour of serum to be explained? The explanation would prove a difficult matter if it were assumed that pancreatic trypsin, which is the basis of the commercial preparation used (Liquor trypsin co.) were a pure enzyme. The pancreatic secretion however contains two proteolytic enzymes, protease breaking proteins down to polypeptides and increasing predominantly the free acidity of the digest, and peptidase, hydrolysing the polypeptides to amino-acids and producing a rise in "formol" acidity.

If this view is correct, the behaviour of serum becomes more easy to explain. It does not exert its action against trypsin as a whole, but only against the protease portion of it. The inhibition of protease will, by preventing the liberation from the proteins of a suitable substrate for peptidase, produce an apparent inhibition of the latter. There does not, however, appear to be any necessity for assuming that the initial depression of "formol" acid formation is due to an antipeptidase action of serum rather than to a shortage of substrate consequent on protease inhibition.

But why should "formol" acid production in the unheated serum tubes exceed that in the heated tubes? There are two possible explanations. Serum contains either a peptidase or a peptidase-accelerator. The most likely explanation is the existence of a serum-peptidase, which has been reported by various workers, including Jobling *et al.* [1912] who report that serum-peptidase is destroyed by heating at 70° and [1915, 2] that "serum-peptidase is not influenced by antitrypsin." Hence, when protease has recovered from the inhibition by serum, the peptidase of the unheated serum will reinforce that of the pancreatic trypsin, and so produce "formol" acid at a rate which will outstrip the "formol" acid production in the tube containing heated serum (where pancreatic peptidase acts alone, the serum-peptidase being destroyed by heat).

(*d*) In a word, the behaviour of serum to pancreatic trypsin can be explained by the presence of two factors, firstly, an antiprotease factor, and secondly, peptidase.

This conclusion is of some importance for at least two reasons. In the first place the evidence justifying it confirms in a novel yet definite manner the dual nature of trypsin; secondly, this evidence, by showing that it is the protease only (the portion forming free acid) which serum inhibits, directs serious criticism against those methods of measuring "antitrypsin" which estimate together the products of both protease and of peptidase actions. The methods thus open to criticism include those of Bayliss (electrometric), and Robertson (refractometric) though not of Folin (uncoagulated N). The error involved will depend on the period of incubation used as well as the strength of serum-peptidase present; the extent of the error will be appreciated in the following example.

	Course of digestion of heated and unheated sera								
	8 hrs.			27½ hrs.			95 hrs.		
	F	For	T	F	For	T	F	For	T
Unheated sera	0·03	0·06	0·09	0·13	0·19	0·32	0·23	0·41	0·64
Heated sera	0·22	0·10	0·32	0·27	0·17	0·44	0·27	0·28	0·55

F = Free acid. For = "Formol" acid. T = Total acid.

Figures are cc. $N/100$ acid in 0·1 cc. of incubated fluid.

It will be readily seen that there is a considerable discrepancy between the degree of inhibition as measured from the free acid and that measured from the total acid. Thus:

	% inhibition (or "antitryptic index")		
	8 hrs.	27½ hrs.	95 hrs.
From free acid figs.	86	52	15
From total acid figs.	72	27	−16*

* *i.e.* 16 % acceleration.

The methods criticised will therefore give too low a value for the index of inhibition.

The kinetics of serum antitrypsin.

(*a*) *The kinetics of recovery.* The Dastre phenomenon was only obtained when the concentration of serum was suitably adjusted to the concentration of trypsin. On the other hand, it was found that (1) when the concentration of trypsin was greatly in excess of the serum the latter failed to produce any appreciable inhibition; (2) when the concentration of serum was greatly in excess of the trypsin, the latter completely failed to recover from the inhibition. The correlation of the relative trypsin-serum concentration with the manifestation of the Dastre phenomenon is illustrated by Fig. 10. The curves were

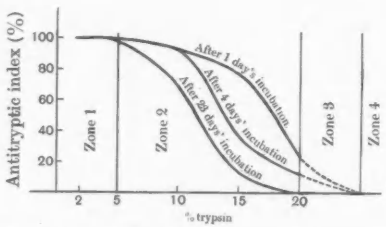

Fig. 10. Inhibition of trypsin (from 2 % to 20 %) by 10 % sheep serum. This illustrates the kinetics of recovery of trypsin from inhibition. The figure suggests a division of the curves into four trypsin zones.

Zone 1. 0 %–5 % trypsin. Here there is no recovery from the inhibiting action of 10 % serum.

Zone 2. 5 %–20 %. Inhibition is initially complete to partial, and recovery is partial to complete.

Zone 3. Trypsin 20 %–25 % (dotted lines); inhibition is initially small, and recovery is complete.

Zone 4. Trypsin over 25 %. Inhibition is absent. No experiments were actually carried out in Zones 3 and 4, which are merely strongly suggested by the character of the Zones 2 and 3, and by the fact established on other occasions that concentrated trypsin successfully resists the inhibiting action of serum.

obtained by plotting the digestion of a series of tubes containing (1) trypsin in concentrations from 20 % to 2 % of the commercial preparation; (2) sheep serum in constant concentration of 10 %, and (3) buffer, p_H 8. A pair of tubes was put up at each concentration of trypsin, one containing unheated serum (tube 1), the other heated (tube 2): each point on the curve was obtained by expressing as a percentage the ratio

$$\frac{\text{total acid in tube 2} - \text{total acid in tube 1}}{\text{total acid in tube 2}}$$

which expresses the degree of inhibition by the unheated serum. The total acid figures were taken because in this case the value of serum-peptidase was practically nil, so that the error involved in including "formol" acid did not affect the nature of the curves obtained, although it would affect the accuracy of the individual antitryptic indexes.

An examination of Fig. 12 shows that (1) in concentrations of 4 % and less trypsin is completely inhibited and does not recover from the inhibition even after 23 days; (2) between 4 % and 20 % trypsin is only partially inhibited and recovers to an extent proportional to its concentration; (3) at 20 % recovery is ultimately complete. From the nature of the curves it would be reasonable to infer that concentrated trypsin completely resists the inhibiting action of serum (dotted lines in Fig. 10).

From the continuous nature of the curves it appears likely that the action of serum is essentially the same at all concentrations of trypsin, varying in degree only, and that therefore the apparently permanent inhibition of weak trypsin is not due to destruction of the trypsin, any more than is the temporary inhibition of stronger trypsin.

(*b*) *The work of Hedin.* Hedin [1906, 1] found that (1) a small amount of serum had a relatively greater effect on trypsin than a large amount; (2) it was impossible completely to neutralise a given solution of trypsin by adding any amount of serum. This result is confirmed by Young [1918] whose results are illustrated in Fig. 11: this curve shows that excess of serum has no effect on the residual activity of trypsin.

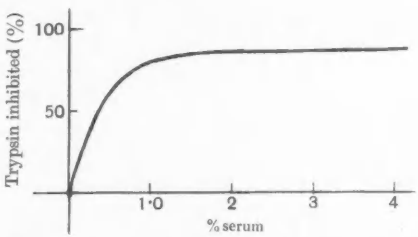

Fig. 11. Relationship between serum and inhibited trypsin [experiment by Young, 1918, p. 503, sheep]. This illustrates: (1) that degree of inhibition is a logarithmic function of amount of inhibiting serum (thus suggesting that inhibition is an adsorption phenomenon); (2) inability of serum to achieve 100 % inhibition of trypsin, the reasons for which are discussed in the text.

Hedin thus shows that a mixture of trypsin and excess of serum possesses both antitryptic and tryptic actions, *i.e.* trypsin and antitrypsin can co-exist when the latter is in excess, each exerting an independent action on added substrate or enzyme respectively.

I have not been able to confirm the common findings of Hedin and Young that excess of serum will not entirely inhibit a given amount of trypsin. Fig. 10 shows that 4 % and 2 % trypsin were completely inhibited by 10 % of serum. The cause of the discrepancy is difficult to explain, but may be due to the difference in the methods employed in studying the kinetics of trypsin. Hedin and Young combined fairly concentrated trypsin in constant amount with increasing amounts of serum: hence all flasks contained a fairly active peptidase together with a large excess of caseinogen added as substrate. Caseinogen it is stated by Jobling and Petersen [1914, 4, p. 152] is an exception to the rule that peptidase does not split proteins; furthermore, peptidase is uninfluenced by the antiferment (Jobling).

(*c*) *Analogy with charcaol.* To Hedin [1906, 2] is also due the discovery that charcoal inhibits trypsin in a manner kinetically very similar to that of serum. Thus the amount of trypsin bound by charcoal follows the same law as the binding of trypsin by serum: small amounts of charcoal bind relatively more trypsin than larger amounts.

This relationship, which can be expressed by the equation $x = ky^n$ (where x = amount of trypsin bound, and y = amount of active charcoal), is a logarithmic one, and is readily accounted for by assuming that the mode of action of charcoal is one of adsorption of the trypsin. Hence Hedin on the basis of kinetic similarity concluded that serum also inhibited trypsin by the process of adsorption.

The behaviour of charcoal throws some further light on the adsorptive process. Hedin showed that although a trypsin solution filtered after treatment with excess of charcoal was quite inactive, yet an unfiltered trypsin-charcoal mixture incubated with caseinogen was definitely and invariably active, though most of the trypsin remained inhibited. Hedin concluded that in the presence of subsequently added substrate charcoal did not retain all the trypsin adsorbed, a small portion of it being taken up by the substrate. In a similar manner might be explained the impossibility of totally inhibiting a solution of trypsin by means of serum, although it is impossible actually to demonstrate the similarity since trypsin cannot be removed with its inhibitor from a trypsin-serum mixture in the simple manner possible with charcoal.

(*d*) *Robertson's formula.* Robertson [1918] using the refractometric method, examined quantitatively the inhibition of trypsin by serum and arrived at the formula

$$\frac{T}{A(1-T)} = C$$

where T = fraction of trypsin inhibited;

A = concentration of serum present;

C = a constant representing the antitryptic value of the serum used.

Robertson claimed that his constant was an absolute measure of the inhibiting power of serum, being independent of the concentration of serum used. An examination of his results, however, shows that the fraction $\frac{T}{A(1-T)}$ tends to be constant only for mixtures containing more concentrated serum (10 % to 33 %) and even then the variation in C is too considerable (5·87 to 6·95 for a single serum) to permit of the value of C being used as a characteristic figure for the inhibiting power of serum.

Where approximate results only are required, however, the constant is a useful expression of antitryptic power, and sufficiently accurate, provided the concentration of serum in the mixture is not too small. By this method Hanson [1918] was able to show that injections of trypsin had no influence on the globulin/albumin ratio of serum, although the antitryptic index (as measured by C) rose to three times the normal value.

(*e*) *The specificity of the inhibiting action of serum.* Von Eisler [1905] found that human serum did not inhibit human pancreatic trypsin any more than it inhibited pig trypsin, while Weil [1910] found that serum actively inhibited the vegetable protease papain. Serum, it follows, does not exert any biologically specific action on trypsin, but inhibits all proteases alike. Apparent specificity may be shown by serum nevertheless, as the work of Young [1918] proves. He found that whereas dilute serum equally inhibited two samples of equally active trypsin obtained from the same source, the inhibition was different when the concentration of serum was increased. Fig. 12 illustrates

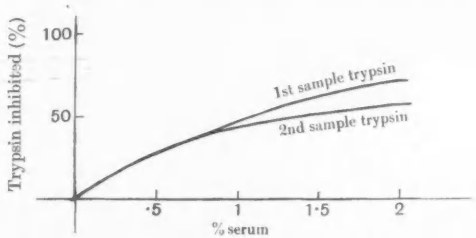

Fig. 12. Inhibition of equally active samples of ox pancreas [experiment by Young, 1918, p. 506, Exp. IV]. This shows that samples of trypsin equally inhibited by weak serum may not be equally inhibited by stronger serum.

this result, and Fig. 13 shows the result of another of Young's experiments in which equally active samples of fresh and commercial trypsin are compared. Young was unable to account for the preferential action shown by serum for certain trypsins, though this preference was not shown in all the experiments carried out: he did show, however, that the presence of zymoids could not account for the phenomenon.

His findings are important in showing the danger of attributing all variation in antitryptic action to the serum alone without first determining the uniformity of the trypsin solutions used.

The influence on serum of injections of trypsin.

A careful examination of the properties of serum-antitrypsin and of the various methods employed in measuring its value has led me to the conclusion that the failure of workers to obtain a rise in antitryptic value of serum following injections of trypsin could adequately be accounted for by one or more of the following circumstances[1].

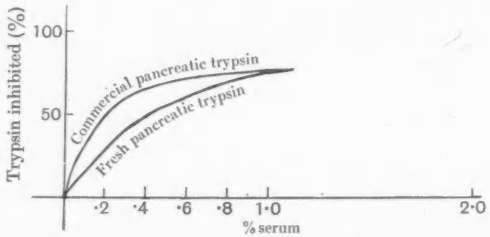

Fig. 13. Inhibition of equally active samples of fresh and commercially prepared trypsin [experiment by Young, 1918, p. 505, Exp. 1]. Here weak serum inhibits the two samples differently, the difference diminishing as the serum becomes stronger.

(*a*) The antitryptic power of serum is not constant in value, but follows a swinging course which in the normal animal is often determined by definite physiological conditions. Thus Jobling *et al.* [1915, 1] found a definite rise a few hours after a meal, a result which I have confirmed.

(*b*) It is generally agreed that the variations in antitryptic value are not very great. In Hanson's experiments [1918] the rise never exceeded three times the normal index. Consequently if the serum were antitryptically at its peak, an injection of trypsin would fail to produce a further rise in index.

(*c*) Hanson records that when the index returns to normal after a rise due to a trypsin injection, further repeated injections fail to produce a second rise. It is probable that such a refractory condition of serum might arise from physiological conditions other than the results of trypsin injections.

(*d*) The choice of method has a very important effect on the results obtained [Fine, 1930].

Experimental demonstration of the rise in antitryptic index following injections of trypsin. The results of experiments carried out with three guinea-pigs are graphically represented in Figs. 14, 15 and 16.

The method of obtaining the antitryptic index may be considered open to criticism since, in the technique employed, the substrate used is the serum itself and must therefore vary with each specimen taken: secondly, it may be objected that since substrate is not present in excess, the extent of hydrolysis

[1] Among those who failed were Doblin [1909], Rosenthal [1910], Weil [1910], Young [1918], and Pozerski [1909] (using papain instead of trypsin). Positive results, on the other hand, were obtained by Achalme [1901], von Berman and Bamberg [1908], Jochman and Kantarovitch [1908], Meyer [1909], Jobling, Petersen and Eggstein [1915, 2] and Hanson [1918].

in 24 hours cannot be considered as representing the activity of the enzyme present.

I would answer these objections as follows.

(*a*) The index is calculated from the ratio of digestion of a given amount of serum-protein to the digestion of the same amount but in the absence of

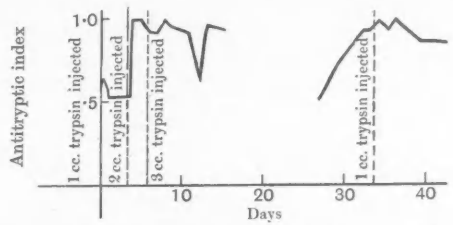

Fig. 14. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *T*. This shows a rise in index immediately or shortly after each injection of trypsin. The guinea-pig was bled daily without definite relationship to the time of feeding, this leading to apparently spontaneous fluctuations in the curve.

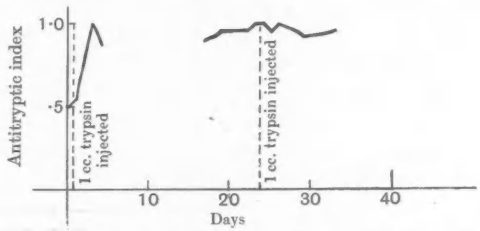

Fig. 15. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *U*. After the first injection the index rises and remains high throughout the experiment.

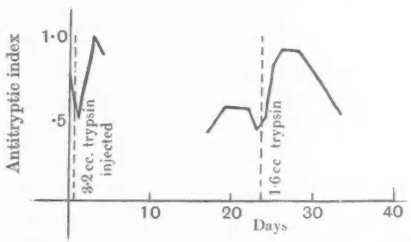

Fig. 16. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *V*. Both injections are definitely associated with a subsequent rise in index. The very large first injection produced a preliminary fall.

any inhibition. Actually of course the index is the difference between this ratio and unity. Hence the actual amount of protein present is not of consequence provided it does not vary greatly from specimen to specimen: in practice the variation was not found to be great.

(*b*) It is true that the acidity measured is not a true measure of the activity of enzyme present: it must be realised, however, that in the method used it is not the activity of the inhibited enzyme that is measured, but the extent to which it has in a given time recovered from the inhibiting action, this degree of recovery being directly related to the degree of inhibition.

An important advantage of the method used is the possibility of distinguishing between the free acid and "formol" acid produced: since the formation of free acid only is opposed by serum, the index is in all cases calculated with reference to free acid formation only.

The three guinea-pigs experimented on were kept under observation for over a month: in each case there was a gap of 14 days during which, as can be seen, no examinations were made owing to an interruption in the investigation.

Guinea-pig *T* did not respond to any extent to the first injection of 1 cc. of trypsin (Benger's), but showed a rise within a few hours of a second injection of 2 cc., which persisted for some time. After a month the index was swinging considerably, with a tendency to rise, which a third injection of 1 cc. maintained. Guinea-pig *U* demonstrated in unequivocal fashion the rise in index in response to an injection of 1 cc. trypsin. The rise was maintained even after 20 days, a second injection having no observable effect. Guinea-pig *V* also responded to a first injection, although the rise was here preceded by a definite fall in index, accompanied by symptoms of collapse (immobility of many hours' duration). In 17 days the index had returned to a lower level, and again responded to an injection (of half the first dose): the rise was maintained for a week. A fall in index immediately following a large injection of trypsin seemed to be associated with symptoms of collapse. Thus guinea-pig *Q* became immobile, following an injection of 2·5 cc. of trypsin, and, although recovery set in in 20 minutes, the index fell from 0·6 to 0·4 in 5 hours, and death ensued in 22 hours. In this case the trypsin was not neutralised, though in all other cases it was brought to a p_H of 8·3 before injection: the route in all cases was intraperitoneal. It will be seen that although guinea-pigs *T*, *U* and *V* responded similarly to adequate injections of trypsin, they differed in their subsequent condition, the index of *T* tending to remain high, but swinging, that of *U* remaining persistently high, while that of *V* tended to return to normal in a few days.

The nature of serum-antitrypsin.

There are two questions which it is necessary to answer if the nature of serum-antitrypsin is to be known: firstly, what is the substance in serum responsible for the inhibition of trypsin? secondly, what is the mode of action of this substance on trypsin?

(1) *The antitryptic substance.* The three main theories as to identification of the antitryptic substance are described below, and their relative merits then discussed: the antibody theory is treated as a variation of the protein theory.

(*a*) *Amino-acid theory.* Bayliss first showed [1904] that the end-products of tryptic action retard the action of trypsin: Abderhalden and Gigon [1907] confirmed the fact that trypsin was retarded by amino-acids. On the strength of such observations Rosenthal [1910] suggested that serum-antitrypsin owed its action to the amino-acid content of serum. Walters [1912] pointed out that the action of amino-acids is too slight to account for the behaviour of serum. More recently Hussey and Northrop [1923] suggested that the polypeptides formed by tryptic action are the source of serum-inhibition. No serious proof has been offered for either of these theories, and the occurrence of the Dastre phenomenon contradicts them emphatically: end-products of tryptic action cannot reasonably account for an antitryptic action which is at its maximum at the commencement and progressively diminishes.

(*b*) *Lipoid theory.* Schwartz first showed [1909] that extraction with ether lessened the antitryptic power of serum, and considered this as proof that serum owed its antitryptic action to its lipoid content. His findings were confirmed by Sugimoto [1913], and the lipoid theory in a modified form was strongly urged by Jobling and his colleagues in a series of papers entitled "Studies in ferment action" [1914, 1–4]. Jobling submitted evidence showing (1) that unsaturated fatty acids and soaps possessed definite antitryptic action; (2) that saturated fatty acids and soaps possess no such antitryptic action; (3) that the iodination or hydroxylation of unsaturated fatty acids or soaps leads to the loss of antitryptic action: similar treatment of serum leads to the loss of antitryptic action of serum. This is confirmed by Slovzov and Zenophentova [1919]; (4) that although unsaturated soaps do not lose antitryptic action when heated alone, they do lose this action if heated at 70° for half an hour in the presence of serum; (5) that the chloroform extract of serum will exert an antitryptic action if saponified, although without this treatment the extract will not exert such action.

A more careful scrutiny of Jobling's experimental results, however, reveals the following facts: (1) On no occasion is there any record of an unsaturated lipoid exerting antitryptic action without previous saponification. (2) Insufficient regard is paid to the p_H of the enzyme mixtures. (3) The attempt is made to bridge the gap between antitryptic lipoids and serum as regards the effect of heat by showing that unsaturated soaps do lose antitryptic power when heated in the presence of serum. From Jobling's figures, however, it is clear that loss of antitryptic power of the unsaturated soap takes place on mixing with serum in the cold, and that heat merely increases the loss: acidity of the soap-serum mixture also increased the loss.

That the lipoids of serum have any connection with the antitryptic action of the latter is contradicted by the work of Cobliner [1910], who found that dry serum retains its antitryptic action after extraction with chloroform or other fat solvents.

Finally, I have shown that the nature of the inhibition produced by serum is essentially different from that produced by unsaturated soaps. The initial

effect of 0·5 % of sodium oleate is to stimulate trypsin, which after 24 hours shows lessening of activity. Serum, on the other hand, initially exerts its maximum inhibition on trypsin, which may ultimately recover completely. This contrast is illustrated in Fig. 17 which represents one of several experiments carried out.

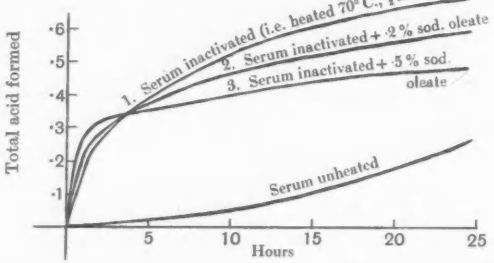

Fig. 17. Comparison between inhibition of trypsin by unsaturated soap and inhibition by serum. This shows that sodium oleate initially stimulates and ultimately depresses trypsin, whereas serum exerts an initial inhibition from which trypsin progressively recovers.

These curves were obtained by plotting the course of digestion of the following four tubes, the usual Sørensen's technique being followed.

	Tube 1	Tube 2	Tube 3	Tube 4
Serum	0·2	0·2 inactivated	0·2 inactivated	0·2 inactivated
Trypsin 50 % (neutralised)	0·4	0·4	0·4	0·4
Buffer (p_H 8)	0·9	0·9	0·9	0·9
Sodium oleate 2 %	0·0	0·5	0·2	0·0
Distilled water	0·5	0·0	0·3	0·5

Figures represent cc.

(*c*) *The protein theory.* It has been definitely shown that the antitryptic action of serum is associated with the protein fraction [Landsteiner, 1900; Cathcart, 1904; and others], the albumin fraction being more antitryptic than the globulin.

Fujimoto [1918] obtained crystallised albumin and found it to be highly antitryptic. He admitted the possibility of some other constituent of serum entering into the composition of the crystals however, and on the whole considered that the antitryptic action was due to the serum-proteins and possibly some other undetermined factors in addition. Oppenheimer [1913] came to the similar conclusion that antitryptic action of serum was due to multiple causes, of which the peculiar configuration of the protein molecule was one [Oppenheimer and Aron, 1903]. Teale and Bach [1920] concluded that the nature of the serum-proteins was the cause of the antitryptic action of serum.

Beaton [1922] found however that the antitryptic index may rise without any change in the albumin content of serum, and in some cases the rise may even be accompanied by a fall in serum-albumin.

There is therefore some additional factor in the action of serum besides

the presence of protein, and this factor is in all probability a physical one, namely the degree of dispersion of the proteins.

(2) *Mode of action of proteins.* The classical work of Hedin [1906, 1, 2] confirmed by Hata [1909], Bayliss [1923], Young [1918] and others, has shown that the mechanism of inhibition by serum is one of adsorption of trypsin by the serum-proteins. Such a mechanism is perfectly reconcilable with Beaton's findings since the amount of adsorption depends not only on the amount of the adsorbing colloids, but also on their degree of dispersion. Thus the antitryptic index of serum might increase without the concentration of proteins necessarily increasing, for the existing amount of circulating protein, by an increase in its state of dispersion, can give rise to a greatly increased surface for adsorption, and therefore to a heightened capacity to adsorb trypsin. It can be shown by a simple calculation that when any particle undergoes division into n smaller particles, the total surface of the smaller particles is $\sqrt[3]{n}$ times the surface of the parent particle. Hence, if the degree of trypsin inhibition can be considered as directly proportional to the surface presented by the colloidal particles of the serum-protein, then the antitryptic index can be doubled either by doubling the amount of protein without altering the average size of colloidal particle, or by the formation of eight colloidal particles from each original one without altering the total amount of circulating protein.

The mechanism of adsorption would also serve to explain one important difference between trypsin inhibition and the inhibition of other enzymes by serum—namely the capacity of trypsin ultimately to recover from the inhibition, there being no such recovery reported for other enzymes. As Bayliss points out [1923], the adsorbed trypsin is able slowly to attack the serum-proteins adsorbing it until the latter are entirely hydrolysed and the trypsin is thus liberated.

When another enzyme such as invertase is inhibited by serum then, on the assumption that the inhibition is also due to adsorption by serum-proteins, it would not be expected that recovery should take place, since invertase would be unable to hydrolyse the adsorbing bodies. In my own experiments with invertase I found that inhibition was not recovered from; thus an invertase solution when unbuffered produced 10 % inversion of 2 % cane sugar in 24 hours and 30 % inversion in 3 days, while with a similar unbuffered solution in the presence of 10 % serum no inversion took place after 3 days.

Adsorption however is not a complete explanation of serum-antitrypsin; since all enzymes before exerting their specific action must be adsorbed by their respective substrates it still remains to be explained why trypsin should be inhibited when it is adsorbed by serum-proteins, although it acts freely when adsorbed by other protein substrates.

Discussion of the antibody theory.

There is no doubt that kinetically the action of serum on trypsin resembles that of antiserum on toxin: in both cases the amount of serum required is not proportional to the amount of substance neutralised, the ratio of serum required increasing very considerably as neutralisation of the substance is approached. The relationship is represented by a logarithmic curve such as satisfies the requirements of an adsorption phenomenon.

It may therefore be said that immunological antibodies and serum antitrypsin resemble each other in the following respects.

(*a*) Both are proteins acting by means of adsorption.

(*b*) They can be increased in amount by injections of the substances they antagonise.

(*c*) Both are normally present in serum.

On the other hand the following differences between them exist.

(*a*) Immune antibodies are associated with the globulin of serum: antitrypsin with the albumin chiefly.

(*b*) The injection of antigens can lead to increase of immune bodies to the extent of thousands of times the normal amount: whereas even an active response to trypsin injections will only treble the normal antitrypsin content.

(*c*) The immune antibodies are highly specific, increased capacity to neutralise a particular toxin being attended by a low neutralising power for other toxins: there is never any marked variation in the power of serum to inhibit the various enzymes normally inhibited by it.

The antibody theory of trypsin therefore amounts to little more than an expression of the fact that both antitoxic action and antitryptic action are adsorption phenomena in which the adsorbing bodies are proteins.

On the occurrence of non-amide linkages in the protein molecule.

In examining the meaning of the Dastre phenomenon, the newer knowledge of the heterogeneous nature of trypsin and erepsin was ignored in order to avoid complication of the issue: in any case it still remains true that the proteolytic enzymes can broadly be divided into two groups—one attacking chiefly the protein molecules and the larger polypeptides, and the other attacking mainly the smaller molecules.

The conclusion that serum behaved as an antiprotease was an expression of the fact that one phase only of the action of trypsin was inhibited. If the action of trypsin on a protein be examined by Sørensen's method it will be found that at the commencement free acid production predominates over formol acid, but that ultimately "formol" acid overtakes free acid.

Since the first action of trypsin is to hydrolyse the protein molecule into polypeptides, the predominance of free acid formation may be correlated with the power to attack the protein molecule, while predominance of "formol" acid may denote hydrolysis of the earlier products of activity. This correlation, which has hitherto been assumed, is borne out by the behaviour of pepsin,

the hydrolysis of proteins by which to the peptone stage only is correlated with the preponderance of free over "formol" acidt hroughout the period of digestion.

Strictly speaking however it cannot be assumed that free acid formation is the sole activity of the enzyme attacking the protein molecule since, as Haldane points out [1930], the specificity of the proteolytic enzymes is not absolutely fixed, each enzyme being capable of effecting a number of allied hydrolytic reactions. Consequently the inhibition of free acid formation does not necessarily prove the inhibition of protease, but denotes the suppression of one form of activity of protease.

The significance of free acid formation. Plimmer pointed out [1908] that in addition to the carbamic linkage —CONH— there may be present in the protein molecule (1) the arginine linkage —CH—NH—C—; (2) the diketo-piperazine linkage; (3) among the hydroxy-acids, the ether, ester, and anhydride linkages.

He did not consider it proved that such linkages did exist, however, and even quite recently Gränacher [1929], who believes the presence of the ester linkage highly probable in proteins, considers definite proof of this wanting.

It seems to me that the formation of free acid during hydrolysis of serum-proteins is proof of the existence of non-amide linkages. Clearly the hydrolysis of —CONH— by forming carboxyl- and amino-groups, cannot increase free acidity, but will increase "formol" acidity.

Free acid could, however, be produced by hydrolysis of esters; since this is usually the function of special enzymes (lipase and phosphatase) it is necessary to eliminate the possibility of free acid formation being due to them. In my own experiments this can readily be done.

(1) The commercial trypsin used was kept in the ice chest, and at p_H 2, which is destructive of lipase. Even if lipase were present in the trypsin, and were responsible for free acidity by action on serum-fats, the action would have been greater on unheated serum than on heated, since guinea-pig serum possesses a very active lipase [Fine, 1930].

(2) The action of phosphatase can be eliminated if the amount of available phosphate in serum be considered. On the basis for the figure for human serum (0·005 % phosphorus) the maximum amount of free phosphoric acid obtainable from 0·2 cc. of a 10 % solution of serum (the amount used in my titrations) is equivalent to 0·01 cc. *N*/100 NaOH—a negligible value within the range of error of the experiments.

That the function of pepsin is to hydrolyse linkages other than —CONH— is proved by the large proportion of free acid to the total acid developed during hydrolysis of proteins [Wigglesworth, 1928]. There is also more definite evidence of this function. Thus Harris showed [1923] that —SH groups were unmasked during peptic digestion, and Abderhalden and Schwab [1930] showed that pepsin completely hydrolysed dileucylthyroxine, although one of the leucine molecules was combined as an ester. Haldane suggests that in

the latter instance the ester linkage was split by a lipase present, but it is unlikely that lipase would act at the optimum p_H of pepsin.

It is very significant that in a list of 53 polypeptides, of which 29 were hydrolysed by trypsin and 24 were not, all those containing tyrosine (four), cystine (two) and *iso*serine (one), *i.e.* the only amino-acids with potentialities for other than —CONH— linkages, were in the hydrolysable group [Plimmer, 1908]. There is no mention however as to whether free acid increased during any of the hydrolyses, and therefore the nature of the linkages is not proved.

Serum antitrypsin as a resistance of the non-amide linkages of serum-proteins. If free acid formation is admitted to be the result of hydrolysis of other than —CONH— linkages, then the inhibition by serum-proteins of free acid formation must be due to a special resistance of these other linkages, a resistance which is overcome by heating at 70° for half an hour. This effect of heat is not due to coagulation, since serum heated with buffer at p_H 8 became only faintly turbid, yet lost its antitryptic action. Possibly the molecules adjoining the special linkages are stereochemically antagonistic to the action of trypsin, but are sterically altered by heat. Such stereochemical specificity is met with throughout enzyme reactions, one of the simplest examples being the resistance of glycyl-*d*-leucine, but not glycyl-*l*-leucine to hydrolysis by yeast-erepsin [Abderhalden and Handovsky, 1921]. That proteins can become less hydrolysable by trypsin as a result of stereochemical alteration has been shown by Dakin and Dudley [1913] who found that partial racemisation of caseinogen by alkali (the rotary power falling to 60 %) made it resistant to trypsin; Lin, Wu and Chen [1928] however found that resistance, although marked, was not complete.

But serum not only resists trypsin: it adsorbs it also, rendering it unavailable for other substrates present. The complete mechanism of serum-antitrypsin might therefore be conceived as

(*a*) an adsorption, at the ester and other non-amide linkages, of those constituents of trypsin capable of attacking them;

(*b*) a resistance to hydrolysis on account of neighbouring stereochemical influences;

(*c*) a breakdown of the resistance, whether through stereochemical changes or other causes.

Conclusions.

1. The influence of serum on enzymes can be divided into the following categories.

(*a*) *Serum contains the enzyme.* Here an apparent acceleration is produced (*e.g.* diastase).

(*b*) *Serum accelerates an enzyme, although it does not contain it.* This is true acceleration (*e.g.* invertase at p_H 4).

(*c*) *Serum contains the enzyme, but accelerates it more than is accounted for by this fact* (*e.g.* lipase).

(*d*) *Serum inhibits the enzyme.*

2. The effect on invertase varies uniformly with p_H, at certain values of which inhibition takes place.

3. The action of serum is sufficiently accounted for by the change of p_H produced by the serum (*e.g.* pepsin) or by the adsorption of the enzyme by the serum proteins (*e.g.* trypsin), or by both factors (most other enzymes).

4. A comparison between serum-antitrypsin and a typical Ehrlich antibody such as antitoxin shows that they resemble each other in the way they act on trypsin and toxin respectively—*i.e.* by adsorbing them—and in their increase following injections into the animal body of the respective "antigens": nevertheless the increase in antitrypsin is trivial and difficult to obtain compared with the great and constant increase in antitoxin.

5. The inhibition of trypsin by serum is recovered from if digestion is continued. An analysis of the free and "formol" acid figures shows that the inhibition is directed against free acid formation, and that the initially low "formol" acid production is merely the result of shortage of substrate for the peptidase of trypsin.

6. This phenomenon of recovery rules out the lipoid and the amino-acid theories of serum antitrypsin.

An examination of the evidence makes it clear that the proteins are the bodies responsible for the antitryptic action of serum, and that they act by adsorption.

7. The formation of free acid during tryptic and peptic digestion can only be the result of the hydrolysis of other than —CONH— linkages when the presence of other ester-hydrolysing enzymes is eliminated. The behaviour of serum suggests that it is at the non-amide linkages that adsorption of the inhibited enzyme takes place, while the resistance itself may be due to an unfavourable stereochemical configuration.

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LXXVI. THE TOTAL CARBOHYDRATE CONTENT OF ISOLATED FROG MUSCLE.

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Introduction.

There are two methods in general use for the determination of the total carbohydrate content of muscle. In one [Parnas and Wagner, 1914; Parnas, 1921] alcoholic and aqueous extracts of the tissue are made, glycogen being estimated in the residue and soluble carbohydrate in the extracts. In the other [Loewi, 1918; Lesser, 1920; Meyerhof, Lohmann and Meier, 1925] the whole muscle is hydrolysed in dilute acid and the glucose formed is estimated by its reducing power. Both are liable to error since it is difficult to remove non-carbohydrate reducing substances completely. The second method gives higher values than the first. Lesser considers that this is due to incomplete extraction of a dextrin. Parnas and Meyerhof *et al.* attributed it rather to the inclusion of non-carbohydrate reducing substances split off from protein during hydrolysis and not precipitated by mercuric acetate. Bissinger [1926] introduced an adaptation of the Van Slyke [1917] method in which sugar is recovered quantitatively from a copper sulphate-lime precipitate. He and Lesser [1926] found that when this technique was used for the estimation of total carbohydrate in the mouse the extraction and hydrolysis methods gave the same results. The same procedure has been applied to the estimation of alcohol-soluble carbohydrate in nerve by Holmes and Gerard [1929] and in muscle by Anderson and Macleod [1930]. Ochoa [1930] in estimating the total carbohydrate content of frog muscle later dispensed with the separation of sugar by copper-lime precipitation and relied on the complete removal of protein and non-sugar reducing substances by West, Scharles and Peterson's [1929] method. The results of the present work show that although neutral mercuric sulphate does give lower results than mercuric acetate or chloride, copper-lime precipitation will still further diminish the reducing value of the filtrates.

The present investigation is an attempt to apply a modification of Bissinger's method to the estimation of the total carbohydrate of frog muscle and to compare the results with the values found for the glycogen and alcohol-soluble carbohydrate in resting muscles, in muscles incubated in phosphate and bicarbonate buffers and in muscles poisoned with sodium monoiodoacetate.

There is some evidence that a carbohydrate intermediate in complexity between glycogen and glucose may exist in muscle. Laquer [1914, 1921] found that in a phosphate buffer solution the total yield of lactic acid exceeds the initial content of glycogen and can usually be accounted for by the lactacidogen content. Simpson and Macleod [1927] found that the breakdown of glycogen in a muscle frozen in liquid air and allowed to thaw was much more rapid than the formation of lactic acid. Anderson and Macleod [1930] have shown that on keeping after death the glycogen content of mammalian muscle remains steady, or may increase, whilst the lactic acid content increases. Lohmann [1926] has found a trisaccharide among the products of action of muscle extract on glycogen. This trisaccharide was not fermentable by yeast and did not give rise to lactic acid. More recently Barbour [1930] has isolated a trisaccharide formed as the sole product of the hydrolysis of glycogen by muscle extract at p_H 6·3. If such a trisaccharide does exist in muscle it might well escape estimation as an alcohol-soluble carbohydrate and would almost certainly be destroyed, in part at least, by the drastic alkaline hydrolysis required for the glycogen estimation.

The method employed in this work should include such a compound, whilst non-carbohydrate reducing substances should be largely, if not entirely, excluded. It was hoped that the solutions made from the copper-lime precipitates would be free from nitrogen, but this was not found to be the case. It is however unlikely that the nitrogen-containing substances are responsible for more than a small part of the reducing value, since after incubation in a phosphate buffer the carbohydrate content of a muscle falls to about 10 % of the initial value, whilst the nitrogen content of the copper-lime precipitates does not alter appreciably, whence it may be assumed that non-carbohydrate reducing substances have not altered in amount. The error from this cause, if the whole of the final reducing value is due to nitrogen-containing substances, will not be above 10 %. Another source of error, acting in the opposite sense, is the incomplete hydrolysis of carbohydrate. Glycogen is hydrolysed under optimum conditions to the extent of 94 % and other carbohydrate, should any be present, is unlikely to give values as high as 100 %; all values in this paper are given as glucose found, without arbitrary correction. In the time allowed all the hexosephosphate will not be hydrolysed, but as the amount present in muscle is small compared with the total carbohydrate, the error from this cause will be insignificant [Meyerhof, 1930]. In some cases, such as in muscles poisoned with monoiodoacetate, where exceptionally large amounts of hexosephosphate are known to be present, special allowance must be made.

The chief difference between the results found in this work and those of previous workers is the much greater difference between the total carbohydrate content and the sum of the glycogen and alcohol-soluble carbohydrate. Glycogen values vary considerably and it is difficult to compare results found by different workers, but the alcohol-soluble carbohydrate values are much lower.

Meyerhof [1920] found as an average value for resting frog muscle, 194 mg./100 g., whereas the average resting value for the present results is 40 mg./100 g. This may be due both to the use of copper-lime precipitation and to the use of absolute alcohol as an extractant in place of 60 % alcohol, which must extract some glycogen as well as other carbohydrate and so make the results for alcohol-soluble carbohydrate too high. The values for total carbohydrate are decidedly higher than those found by other workers, although lower values would have been expected since sugar was separated by copper-lime precipitation and protein was removed after 30 minutes' hydrolysis, the total period of hydrolysis (1·5 hours) being much shorter than that usually allowed. In one experiment the values for total carbohydrate estimated by several variations of the method were compared. When hydrolysis was discontinued at the end of 30 minutes, protein removed by Schenk's method, and the hydrolysis continued for another hour, the total carbohydrate content of the muscle was 0·973 %, if sugar were separated by copper-lime precipitation, but 1·219 % if calculated from the reducing value of the solution before copper-lime precipitation. If protein were removed by West, Scharles and Peterson's method the values were 0·840 % and 1·022 % respectively, the discrepancy being less after copper-lime precipitation. When hydrolysis was continued for 3 hours before removal of protein by Schenk's method the value was 1·071 % after copper-lime precipitation and 1·347 % before; if protein were removed by West, Scharles and Peterson's method the values were 1·202 % and 1·044 % respectively, higher in all cases when protein was not removed before the completion of hydrolysis. The Schenk method was used throughout this work, as when most of the experiments were carried out the superiority of the West, Scharles and Peterson method was not appreciated; the results are therefore probably too high by 10 % to 15 %, a figure comparable with that estimated for the error due to non-carbohydrate reducing substances.

Methods.

Preparation of material. In all the experiments recorded in this paper single isolated frog muscles were used. When working with single muscles it is essential that all comparisons should be made either in simultaneous determinations on the same muscle, or on pairs of corresponding muscles from the two legs of the same frog. The glycogen and total carbohydrate contents of resting muscles from the same frog vary very much, and a totally wrong idea of the changes taking place may result from comparing different muscles or even by taking average figures. The glycogen and total carbohydrate contents of corresponding muscles of a pair from opposite legs of the same frog agree reasonably well (Table VI), and a much truer picture of changes taking place results from confining all the estimations to one pair of muscles.

The frogs were in all cases the large Hungarian variety. They were killed by a cut across the base of the skull, followed immediately by section of the

spine just above the pelvic girdle. The muscles were dissected, weighed on a torsion balance and dropped into Ringer solution at p_H 7·3, containing 0·012 % sodium dihydrogen phosphate and 0·057 % disodium hydrogen phosphate, but no bicarbonate. Oxygen was slowly bubbled through the solution. The muscles were left in the solution for at least 1 hour and usually longer before further treatment. Dulière and Horton [1929] have shown that muscles treated in this way are not subject to the onset of inexcitability, and that their resting phosphagen content is high and inorganic phosphate low. The lactic acid content is also low, on an average 20 mg./100 g., as is shown by the results in this paper. These figures indicate that the muscles are in a resting condition and have recovered from any stimulation or injury inflicted during killing and dissection. This method is suitable only for thin muscles, such as sartorii or semi-membranosi, and cannot be used for thick muscles such as gastrocnemii, as the oxygenated solution will not diffuse into them; where such muscles were used they were treated immediately after dissection without any soaking in Ringer solution.

Estimation of total carbohydrate: extraction and hydrolysis. 2·2 % hydrochloric acid has been found most suitable for the hydrolysis of glycogen and, since glycogen forms the greater part of the carbohydrate of muscle, this strength of acid was used for the hydrolysis of the whole muscle. In the first experiments the muscles were thrown into boiling acid, but later they were ground in ice-cold acid, and extract and residue were hydrolysed together in a boiling water-bath, as changes due to killing are better avoided in this way. The completeness of the extraction of carbohydrate was tested by determining the amount of glycogen in the residue after varying the times of hydrolysis. Protein was precipitated by Schenk's method, the solid residue removed by centrifuging, and glycogen estimated in it. It was found that after 15 minutes most of the glycogen was removed, and that heating for longer than 30 minutes did not remove appreciably greater quantities, so the latter period was adopted as standard. There was always some glycogen left in the residue, and it did not change in amount during the various treatments applied to the muscle.

Hydrolysis for 30 minutes will not hydrolyse all the carbohydrate present in the muscle completely, but it is inadvisable to heat longer, owing to the danger of further protein breakdown. The time necessary for maximum hydrolysis, after removal of protein, was investigated by hydrolysing the protein-free extract, obtained from a number of muscles, in a boiling water-bath and at intervals removing portions for sugar estimation, after precipitation with copper-lime. The results are plotted in Fig. 1. In one experiment (curve b_2), the nitrogen values of the copper-lime precipitates were determined, and it will be seen that they fall off slowly, whilst the sugar values rise sharply to a peak and then fall away. This may be explained by supposing that a part of the reducing value is due to some nitrogen-containing substance which is slowly being destroyed, as is indicated by the decreasing amounts of nitrogen associated with the copper-lime precipitates. In this experiment (curve b_1), protein was

removed by trichloroacetic acid, which may make the sugar values low; although most of the acid is destroyed during the hydrolysis, sufficient is left to interfere with the sugar estimation, and for this reason the method was not used in later work. One hour was adopted as the standard period for the second hydrolysis. The curves plotted in Fig. 1, which are typical of several obtained, show that hydrolysis was complete in this time.

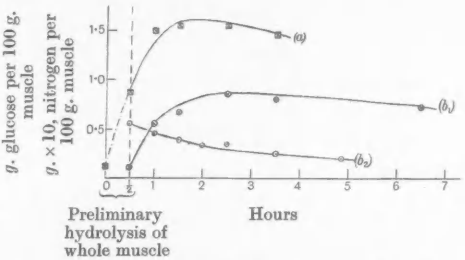

Fig. 1. Sugar formed on hydrolysis of hydrochloric acid extract of muscle.
Exp. (*a*). Sugar formed: protein removed by Schenk's method.
Exp. (*b*) 1. „ „ „ „ „ trichloroacetic acid.
(*b*) 2. Nitrogen content of copper-lime precipitate.

Estimation of sugar in hydrolysed solutions. Sugar was estimated in the solutions after hydrolysis by an adaptation of Bissinger's [1926] method. A further investigation of the conditions of precipitation and recovery of sugar was undertaken, using pure glucose solutions.

Freedom of precipitate from non-carbohydrate reducing substances. Holmes and Gerard [1929] state that creatinine is not precipitated by the copper-lime reagent. In order to test this the effect of treating a solution containing 25 mg./100 cc. of creatinine with copper sulphate and lime was determined. After removing the precipitate the creatinine content was reduced to 80 % of the original. That this was partly due to adsorption or occlusion on the precipitate was shown by dissolving the latter in normal hydrochloric acid and reprecipitating the copper compound with an equal volume of normal sodium hydroxide. By using two precipitations in this way it was possible to recover 95 % of creatinine in the mother-liquor.

In an experiment using a solution formed by the hydrolysis of muscle the nitrogen content of the first copper precipitate was 150 mg./100 g. of muscle. This solution gave a biuret reaction. Solution of the precipitate in normal hydrochloric acid and reprecipitation with normal sodium hydroxide reduced the value to 40 mg./100 g. muscle. This second solution gave no biuret reaction. From these results it was decided to use this method of two precipitations throughout.

Optimum conditions for precipitation of sugar. The solutions were allowed to stand for 30 minutes [Van Slyke, 1917] after addition of copper sulphate and lime, and again for 30 minutes after the second precipitation with sodium

hydroxide. If the alkaline solutions are allowed to stand too long some of the sugar will be destroyed (Table I), in fact some is destroyed in any case, for higher values are obtained when hydrochloric acid is added before copper sulphate and lime, and precipitation is not allowed to take place, the rest of the determination being carried out in the same way. The loss is a constant percentage of the whole and can be corrected.

Table I.

Effect of time of standing on recovery of sugar from copper-lime precipitates. An equal time was allowed after each precipitation.

Time in minutes	Sugar recovered, mg.
25	5·88
30	6·16
35	6·06
40	5·94
45	5·78

The proportion of sugar recovered was constant at any one temperature, and did not vary much with temperature so long as the other conditions remained unaltered (Table II). The solutions were allowed to stand at laboratory temperature, and the factor required was redetermined from time to time, especially if there was known to be any large variation in temperature.

Table II.

Effect of variation of temperature during standing on recovery of sugar from copper-lime precipitates.

Temperature	Sugar recovered, mg.
0°	8·9
18°	8·5
24°	7·9

Proportions of copper sulphate and lime required for complete precipitation of sugar. The amounts both of copper sulphate and of lime used by different workers vary very much. The minimum amounts required were determined for concentrations of sugar up to 0·1 % in a volume of 25 cc. The sugar remaining in solution after addition of increasing amounts of a 10 % solution of crystalline copper sulphate and a 10 % suspension of calcium hydroxide was determined, the precipitated copper compound and excess of lime being removed by centrifuging. The amount of calcium hydroxide was adjusted so that there should always be an excess present after allowing for the formation of calcium sulphate and saturation of the solution with calcium hydroxide. The amount of sugar remaining in solution decreased with increasing amounts of copper sulphate up to 2 cc., but beyond this there was no improvement, the solution after centrifuging had always about 1 % of the original reducing value. Using 2 cc. of 10 % copper sulphate, 0·94 cc. of a 10 % suspension of calcium hydroxide is required for formation of calcium sulphate and for saturation of 25 cc. of solution with calcium hydroxide; in order that there should always be a considerable excess present, 2 cc. were added.

Recovery and estimation of sugar. If a method could be found in which the copper used in precipitating the sugar could be used as the oxidising agent it would simplify the procedure very much. Folin's method [1926] seemed the most likely to be satisfactory, especially as Eggleton (private communication) has shown that it is possible to estimate the molybdenum blue, formed on addition of the acid reagent, by titration with potassium permanganate, instead of colorimetrically. Eggleton found that 1 mg. of glucose required 8·20 cc. of 0·004 *N* permanganate. This method was found very satisfactory when a modification of the alkaline tartrate reagent was made. The large excess of copper did not affect the reaction, and the final titration was sharp enough to make the accuracy of the method sufficient for work on isolated muscles.

The copper precipitate did not dissolve in the tartrate reagent, made up without copper sulphate, owing to the calcium hydroxide still present, but dissolved easily in a slight excess of normal hydrochloric acid, and an aliquot part of this solution was used for sugar estimation. It was not possible to use more than 2 cc. per estimation as Folin's special tubes were used, and as the volume of the solution after dissolving the copper precipitate in *N* hydrochloric acid was not far short of 10 cc., only about $\frac{1}{5}$th could be taken for each determination. The tartrate reagent was made up without copper sulphate and with four times the amount of tartrate used by Folin, since $\frac{1}{5}$th of the dissolved copper precipitate contained four times as much copper as 2 cc. of Folin's reagent. The amount of sodium carbonate was increased from 0·7 % to 7·5 % to allow for neutralisation of hydrochloric acid, the formation of calcium carbonate and for the absorption of the additional carbon dioxide liberated. The amount of sodium bicarbonate was not altered from the 2·0 % in Folin's solution. The ratio of carbonate and bicarbonate formed in this solution was found adequate for buffering changes in p_H due to variation in excess of hydrochloric acid. The accuracy of the estimation was not affected by small excess either of copper or of tartrate, nor did the calcium carbonate precipitated when the two solutions are mixed interfere with the reaction as long as the mixing was efficient.

The final composition of the alkaline tartrate solution using $\frac{1}{5}$th of the dissolved copper precipitate for each determination was as follows:

Sodium potassium tartrate	6 %
Sodium carbonate	7·5 %
Sodium bicarbonate	2 %.

The acid solution was that recommended by Folin:

Sodium molybdate	150 g.	$Na_2MoO_4.2H_2O$ per litre
Phosphoric acid	225 cc. 85 %	acid ,,
Sulphuric acid	150 cc. 25 %	,, ,,
Acetic acid	75 cc. 99 %	,, ,,

Summary of procedure for estimation of total carbohydrate. The muscle, weighing usually 0·15–0·35 g., was ground with sand and 10–15 cc. of ice-

cold 2·2 % hydrochloric acid, and the mixture was hydrolysed for 30 minutes in a boiling water-bath. After cooling an excess of solid mercuric chloride was added, the solution was made up to 25 cc. and allowed to stand. It was then filtered, moist hydrogen sulphide was passed through the filtrate, the mercuric sulphide was filtered off, and the filtrate thoroughly aerated to remove hydrogen sulphide. 15 cc. were hydrolysed for 30 minutes, and after cooling the solution was washed into a centrifuge tube graduated at 25 cc. and at 10 cc.; 2 cc. of a 10 % solution of crystalline copper sulphate were added, and the solution was treated with 40 % sodium hydroxide until the copper was just precipitated. 2 cc. of a 10 % suspension of calcium hydroxide were then added, the solution was well stirred, and water was added to 25 cc. The tube was left to stand for 30 minutes and centrifuged for 3 minutes. The supernatant liquid was poured off and used for lactic acid estimation. The precipitate was dissolved in 5 cc. of *N* hydrochloric acid, 5 cc. of *N* sodium hydroxide were added, the solution was well stirred, and the volume brought back to 25 cc. After 30 minutes the precipitate was separated by centrifuging and dissolved in 3 cc. of hydrochloric acid. Less acid was needed for the second solution as the second precipitate contained less calcium hydroxide. The volume was made up to 10 cc., and 2 cc. of this solution were added to 2 cc. of the tartrate solution in a Folin tube. The sugar solution should be run into the bottom of the tartrate solution, otherwise the calcium carbonate formed greatly impedes the mixing which is absolutely essential for satisfactory results. The tube was placed in a boiling water-bath for 8 minutes, cooled for at least 1 minute in cold water, and the acid reagent was added. When all the cuprous oxide and the calcium carbonate were dissolved, the solution was washed into a large boiling-tube and titrated with 0·004 *N* potassium permanganate. The solution may safely be left for about 30 minutes after addition of the acid reagent, but should be titrated immediately after it has been washed into the tubes for titration. The end-point is not difficult to see after some practice and in a good light, but the titration must be rapid as the blue colour reappears on standing.

When the estimation was carried out in this way, the relationship between the amount of potassium permanganate required and the amount of glucose in solution was linear. The results of estimating pure glucose by this method are plotted in Fig. 2. Curve (*a*) was obtained using only the precipitation and sugar recovery part of the method, curve (*b*) by including the Schenk protein removal technique, starting with addition of hydrochloric acid and mercuric chloride. The mean value for the factor for converting cc. of potassium permanganate into mg. of glucose is 0·1 when the full method is used, but 0·094 when the Schenk procedure is omitted (temperature 16°). Possibly some glucose is lost during aeration of the solution in removing hydrogen sulphide. The recovery of added glucose in a determination of total carbohydrate in muscle was measured and the results calculated, using curve (*b*) Fig. 2, are shown in Table III. The accuracy of the sugar recovery is not nearly as great as in the determinations on pure glucose solutions. The inaccuracies are

probably due to conditions of extraction and hydrolysis of carbohydrate and not to errors in the sugar method.

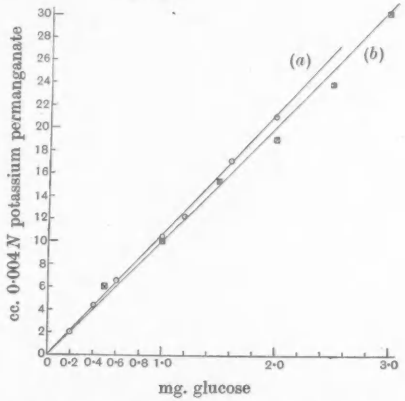

Fig. 2. Titration of glucose recovered from copper-lime precipitate against 0·004 *N* potassium permanganate.
(*a*) Recovery from copper precipitates. (*b*) Including Schenk protein precipitation.

Table III.

3 g. muscle extracted with 100 cc. 2·2 % hydrochloric acid, after 30 minutes' hydrolysis extract filtered and a solution containing 1, 2 and 3 mg. sugar added to portions of 10 cc. Total carbohydrate estimated in each portion.

Sugar added mg.	Carbohydrate found mg. glucose	Glucose recovered mg.	% recovery
0	1·41 1·45 Mean 1·43	—	—
1	2·62 2·39 „ 2·51	1·08	108
2	3·40 3·26 „ 3·33	1·90	95
3	4·53 4·47 „ 4·50	3·07	102

If the reducing value finally measured is due only to glucose, it should be the same when measured by different methods. In order to check this, in an experiment on muscle the reducing value of the second copper precipitate, dissolved in hydrochloric acid, was estimated by Hagedorn and Jensen's method, after removal of copper and calcium [Holmes and Gerard, 1929], as well as by this adaptation of Folin's method. When the results were corrected by a factor found by carrying out the estimations on pure glucose solutions, and necessitated by losses occurring during the copper precipitation, they agreed very well with those found by the Folin method.

Estimation of glycogen. It was necessary to estimate both glycogen and lactic acid in the same muscle, so the muscles were crushed in 10–15 cc. of ice-cold absolute alcohol in well-cooled mortars and washed into cooled centri-

fuge tubes. The tubes were left overnight in the refrigerator to allow complete extraction of lactic acid. The alcoholic extract was separated by centrifuging, the residue was washed twice with absolute alcohol, and glycogen was estimated in it as described in a previous paper [Kerly 1930], the correction for solubility in aqueous alcohol being applied. Sugar was estimated after hydrolysis by the original Hagedorn and Jensen method, by Hanes's [1929] modification or by the Shaffer-Hartmann method. Loss of glycogen is insignificant except perhaps for muscles containing very little glycogen, since the volume of alcohol is very large in relation to the volume of the muscle, and the water content of the muscle has an inappreciable effect on the concentration of the alcohol. Anderson and Macleod [1930] find that for mammalian muscle this procedure is not satisfactory for glycogen estimation, but the method is not quite comparable for the two types of muscle. In the case of mammalian muscle the muscles were cut off the bones and finely minced under alcohol, a process that must take an appreciable time, whilst the frog muscles were allowed to recover after dissection and then dropped into the ice-cold alcohol; within a few seconds at most they were completely disintegrated.

Estimation of soluble carbohydrate. Alcohol-soluble carbohydrate was estimated in the alcoholic extracts of those muscles used for estimation of glycogen and lactic acid. In some cases determinations were made of the initial reducing value only after copper-lime precipitation, in others of the reducing values both initially and after hydrolysis. For convenience the former is described arbitrarily as the alcohol-soluble sugar, the latter as alcohol-soluble carbohydrate. The alcoholic extract and washings were evaporated nearly to dryness, water was added and a few drops of benzene, and heating was continued to remove the last traces of alcohol [Boyland, 1928]; the solution was then made up to 25 cc. 15 cc. were used for determination of sugar and 10 cc. for carbohydrate. Copper sulphate and lime were added and sugar was estimated as described above. For alcohol-soluble carbohydrate hydrochloric acid was added to make 2·2 % and the solution was hydrolysed for 3 hours in a boiling water-bath, sugar being estimated in the hydrolysate. The insignificant amounts of fat and protein extracted by the absolute alcohol were not removed. The conditions chosen were those most suitable for hydrolysis of glycogen, but the time is quite inadequate for hydrolysis of hexosemonophosphate which should be included both in the determination of sugar and of carbohydrate, since it is carried down by the copper-lime precipitate [Embden and Zimmermann, 1924].

The values found for both alcohol-soluble sugar and carbohydrate were much lower than those usually quoted, probably owing to the failure of most workers to remove reducing substances other than sugar. The average values found for resting muscle were 20 mg./100 g. for sugar (of the same order as those found by Anderson and Macleod for mammalian muscle, using a copper precipitation method), and 40 mg./100 g. for carbohydrate, the variation from the mean being large in both cases. The value for alcohol-soluble sugar may be compared with that found for hexosemonophosphate by Embden and Jost

[1928] and by Eggleton and Eggleton [1929], who found a value of 5–8 mg./100 g. P corresponding with 30–40 mg./100 g. hexose. If it be assumed that hexosemonophosphate gives a reducing value corresponding to 50 % of its hexose content [Meyerhof, 1930], then 20 mg. of alcohol-soluble sugar represents 40 mg. of sugar as hexosemonophosphate. The agreement suggests that the alcohol-soluble sugar of muscle is hexosemonophosphate, but as absolute alcohol may not extract all the lower carbohydrate [Holmes, 1929] there may be other reducing sugars present. Neither Embden and Jost nor Eggleton and Eggleton used absolute alcohol as an extractant, so their results are not low for this reason. The reducing value of the alcoholic extracts after hydrolysis is approximately doubled, but hexosemonophosphate is only hydrolysed to the extent of 20 % during 3 hours in dilute acid [Meyerhof, 1930] so that there must be other carbohydrate constituents present in the alcoholic extract of muscle.

The accuracy of individual values for both alcohol-soluble sugar and carbohydrate is not high, but the values taken as a whole indicate approximately the amount present, and whether any gross changes are taking place during activity of the muscle.

Estimation of lactic acid. The method used depended on whether the muscles were used for determination of total carbohydrate or for glycogen and alcohol-soluble carbohydrate.

In a hydrochloric acid extract of muscle. When estimating total carbohydrate the lactic acid was extracted during the preliminary 30 minutes' hydrolysis. Experiments with a solution of zinc lactate in hydrochloric acid showed that none of the lactic acid was destroyed either during prolonged boiling, as long as efficient condensers were used, or during aeration to remove hydrogen sulphide. A measured volume of the supernatant fluid poured off after removal of the first copper-lime precipitate was taken for estimation of lactic acid. A modification of Friedemann, Cotonio and Shaffer's [1927] method was used, in which manganese sulphate was used to catalyse the reaction, but the aldehyde formed was distilled over in a current of steam instead of air. This method gave a mean recovery of 98 % of lactic acid from zinc lactate solutions. The recovery of lactate added to a muscle extract after hydrolysis is shown in Table IV, the results being calculated by means of the recovery factor found for pure zinc lactate solution.

Table IV.

Recovery of lactic acid from zinc lactate solution added to a hydrochloric acid extract of muscle after the first hydrolysis and before removal of protein.

Zinc lactate added mg. lactic acid	Lactic acid found, mg.	Lactic acid recovered mg.	% recovery
0	0·098	—	—
0·422	0·525	0·427	101
0·633	0·741	0·643	101
0	0·114	—	—
0·379	0·504	0·390	103
1·140	1·314	1·200	106

In an alcoholic extract of muscle. When estimating glycogen lactic acid was extracted by the alcohol, the pulverised muscles being left overnight in the refrigerator to ensure complete extraction. Experiment showed that washing with two portions of 5 cc. of absolute alcohol was sufficient to remove all the lactic acid. Lactic acid was estimated, as described above, in the mother-liquor of the first copper-lime precipitate in the portion of the alcoholic extract used for alcohol-soluble sugar estimation. The temperature at which the alcohol is removed must not rise much above 60° as if the solution becomes too hot or if it is evaporated absolutely to dryness, loss is liable to occur in the lactic acid content. In any case the recovery of added lactic acid is not so good as from a hydrochloric acid extract of muscle. The mean of a number of experiments (Table V) was 85 % recovery, and the amounts of lactic acid found were corrected by this figure. The small quantities of fat and protein, which were not removed, were found not to interfere with the estimation.

Table V.

Recovery of lactic acid from an alcoholic extract of muscle, extract divided into two equal portions and 1·03 mg. lactic acid (as zinc lactate) added to one portion, (B), lactic acid estimated as described in text in both portions.

Portion A lactic acid mg.	Portion B lactic acid mg.	Lactic acid recovered mg.	Recovery %
0·247	1·095	0·848	82
0·254	1·187	0·933	91
0·259	1·170	0·911	88
0·229	1·106	0·877	85
0·265	1·173	0·908	88
0·209	1·030	0·821	80
0·129	0·992	0·863	84
0·101	0·925	0·824	80
0·441	1·326	0·885	86
0·305	1·153	0·849	82
			Mean 85

A COMPARISON OF THE CHANGES TAKING PLACE IN THE TOTAL CARBOHYDRATE CONTENT AND IN THE DIFFERENT CARBOHYDRATE FRACTIONS.

The total carbohydrate, glycogen and alcohol-soluble carbohydrate of resting muscles. The total carbohydrate content and the glycogen content of pairs of muscles from opposite legs of frogs are shown in Table VI. The muscles were all in a resting condition, having recovered from dissection in oxygenated Ringer solution. The agreement between the two muscles is not very exact, but can be said generally to fall within 10 %. The variation from one muscle to another in the same frog is considerable and is greater for total carbohydrate than for glycogen.

In order to compare the total carbohydrate content with the glycogen content one muscle of a pair was analysed for total carbohydrate, the other for glycogen. In some cases the alcohol-soluble sugar, and in two cases the alcohol-soluble carbohydrate, was also determined. In those cases where the alcohol-

Table VI.

Total carbohydrate and glycogen content of pairs of muscles from opposite legs of a frog, muscles allowed to recover in oxygenated Ringer solution.

Frog	Muscle	Weight mg.	Total carbohydrate mg. per 100 g.	Glycogen mg. per 100 g.	Difference %
I	S	319	969	—	3
		311	938	—	
I	Sm	413	1110	—	16
		408	1320	—	
I	B	276	868	—	7
		280	929	—	
II	Sm	377	607	—	2
		397	595	—	
II	B	250	460	—	10
		268	513	—	
III	B	175	920	—	9
		174	834	—	
IV	S	310	1310	—	7
		335	1220	—	
V	S	317	—	523	10
		342	—	463	
VI	B	266	—	568	1
		270	—	563	
VI	S	324	—	648	3
		325	—	668	
VII	S	335	—	758	9
		369	—	694	
VIII	B	217	—	571	6
		241	—	539	
VIII	S	305	—	495	8
		317	—	457	
*IX	G	1131	—	675	9
		1169	—	742	
*X	G	1584	—	558	4
		1588	—	578	

Note. In this and subsequent tables S = Sartorius, Sm = Semi-membranosus, B = Biceps, G = Gastrocnemius, Gr = Gracilis.

* Immediately after dissection, no recovery in Ringer solution.

Table VII.

Comparison of total carbohydrate, glycogen and alcohol-soluble sugar and carbohydrate of pairs of muscles, resting, mg. per 100 g. muscle.

Frog	Muscle	Weight mg.	Total carbohydrate	Glycogen	Alcohol-soluble sugar	Alcohol-soluble carbohydrate	Excess total carbohydrate over glycogen
I	S	315	810	591	30	(60)	219
I	Sm	371	1375	915	14	(28)	460
I	B	224	1305	735	34	(68)	570
II	S	239	965	636	—	—	329
II	B	192	995	721	—	—	274
III	S	203	758	656	49	47	102
IV	Sm	352	1775	931	52	92	844

soluble carbohydrate was not determined, an estimated value for this equal to twice the alcohol-soluble sugar content is included in Table VII for comparison, the values so calculated are enclosed in brackets.

The glycogen content is in all cases much less than the total carbohydrate content, the greatest variation is shown where the total carbohydrate is highest. In no case does the alcohol-soluble carbohydrate account for more than a fraction of the excess of total carbohydrate over glycogen. The values for alcohol-soluble carbohydrate may be low, owing to inefficient extraction from the muscle and to incomplete hydrolysis of the carbohydrates present, *e.g.* hexosephosphate, but when every allowance is made for these corrections, it is impossible that the alcohol-soluble carbohydrate can account for the difference between the glycogen and total carbohydrate. The difference also is too great and too regular to be accounted for by the variation in total carbohydrate and glycogen content of two muscles of a pair, and it is much greater than the maximum amount of non-carbohydrate reducing substance likely to be associated with the total carbohydrate fraction. It may in part be accounted for by ribose derived from adenylic acid. Ochoa [1930] states that as much as 100 mg./100 g. muscle may arise from this source. But even taking into consideration both ribose and non-carbohydrate reducing substances the difference is so great that there must almost certainly be some other substance present.

Changes taking place during incubation in phosphate and bicarbonate buffer solutions. The muscles used in these experiments were allowed to recover from dissection in Ringer solution. One muscle was then analysed and used as the resting control, the other was cut finely with a pair of scissors and placed in a test-tube under 1–2 cc. of the buffer solution. The tube was placed in an incubator at 37° for 3 hours, then the cut muscle was analysed in the same way as the control. The buffer solution was added to the extract. So that estimations of total carbohydrate should be comparable with estimations of glycogen and soluble carbohydrate both experiments were carried out on frogs from the same batch.

Phosphate buffer. A mixture containing 95 % $M/5$ disodium hydrogen phosphate and 5 % $M/5$ sodium dihydrogen phosphate was used, p_H 8. The results are shown in Tables VIII and IX. The total carbohydrate and lactic acid changes and some glycogen and lactic acid changes were estimated using autumn frogs. A more extensive set of experiments on the glycogen and lactic acid changes was carried out on two further batches, one of autumn and one of summer frogs.

In all the cases examined nearly the whole of the carbohydrate of the muscle has disappeared, a mean value of 92 mg./100 g. muscle remaining, or 5–14 % of the initial content, with an average value of 9 %. In no case is the increase of lactic acid equal to the decrease of carbohydrate, in all cases except the first a large amount of carbohydrate has disappeared that has not been converted into lactic acid. It is possible that some or all of it has been changed into hexosephosphate, but from the large difference between the carbohydrate unconverted to lactic acid and the residual value, it is not likely that the final

Table VIII.

Change in glycogen, lactic acid, alcohol-soluble sugar and carbohydrate of muscle incubated for 3 hours at 37°, mg. per 100 g.

Frog	Muscle	Weight mg.	Glycogen initial final change	Alc.-sol. sugar initial final change	Alc.-sol. carbohydrate initial final change	Lactic acid initial final change
		(*a*) Phosphate buffer, autumn frogs, same batch as Table IX (*a*).				
I	Sm	210	749	2		10
		212	67 – 682	9 + 7	—	615 + 605
I	S	172	400	0		4
		162	72 – 338	19 + 19	—	341 + 337
II	Sm	164	916	10		10
		161	350 – 566	46 + 36	—	592 + 582
		(*b*) Phosphate buffer, autumn frogs, another batch.				
III	B	246	710			74
		206	102 – 608	—	—	707 + 633
IV	Sm	509	1090			14
		478	60 – 1030	—	—	989 + 975
		(*c*) Phosphate buffer, summer frogs.				
V	S	244	598			17
		—	76 – 522	—	—	700 + 683
V	B	235	569			63
		—	72 – 497	—	—	603 + 540
VI	S	274	555			18
		—	84 – 471	—	—	622 + 604
VI	B	261	605			15
		—	92 – 513	—	—	632 + 617
VII	S	293	588			14
		—	89 – 499	—	—	564 + 550
VIII	S	259	802			5
		—	97 – 705	—	—	703 + 698
IX	B	175	696			9
		—	137 – 559	—	—	559 + 550
		(*d*) Bicarbonate buffer, autumn frogs, same batch as Table IX (*b*).				
X	S	198	915	24	35	44
		186	469 – 446	23 – 1	43 + 8	241 + 197
XI	Sm	181	1150	0	36	26
		192	400 – 750	42 + 42	57 + 21	695 + 669
XI	B	112	951	24	27	21
		112	594 – 357	12 – 12	72 + 45	337 + 316
XII	B	190	754	47	16	56
		189	473 – 281	58 + 11	79 + 63	444 + 388
XIII	Sm	199	1855	20	0	79
		200	799 – 1056	65 + 45	85 + 85	445 + 366

reducing value is all due to hexosephosphate. It is also possible that this final reducing value is due partly or entirely to non-carbohydrate reducing substances, since the copper-lime precipitates were not free from nitrogen. If this excess of reducing substance is converted into a compound allied to lactic acid it should be estimated, in part at least, with lactic acid, though it might possibly be destroyed during the acid hydrolysis.

The amount of carbohydrate which is changed into lactic acid is of the

Table IX.

Change in total carbohydrate, lactic acid and "residual glycogen in muscles after incubation for 3 hours at 37°, mg./100 g.

Frog	Muscle	Weight mg.	Total carbohydrate initial / final / change	Lactic acid initial / final / change	Residual glycogen (i) initial / final / change	Residual glycogen (ii) initial / final / change
			(*a*) Phosphate buffer, autumn frogs, same batch as Table VIII (*a*).			
I	Sm	215	720	24	64	87
		215	98 – 622	640 + 616	80 + 16	103 + 16
I	B	144	582	34		
		114	60 – 522	283 + 249	—	—
II	Sm	473	1610	20	28	39
		490	84 – 1526	1030 + 1010	16 – 12	32 – 7
II	B	322	1092	8	45	64
		304	119 – 973	608 + 600	79 + 34	101 + 37
III	Sm	243	1250	42	*34	*53
		256	85 – 1165	487 + 445	*35 + 1	*55 + 2
IV	B	231	1695	20	*25	*49
		231	108 – 1587	970 + 950	*32 + 7	*65 + 16
			(*b*) Bicarbonate buffer, autumn frogs, same batch as Table VIII (*d*).			
V	Sm	264	1335	38		
		234	831 – 504	486 + 448	—	—
V	B	162	1055	34		
		157	658 – 397	604 + 570	—	—
VI	S	152	826	50		
		154	309 – 517	462 + 412	—	—
VII	Sm	210	1530	55		
		182	527 – 1003	793 + 738	—	—
VIII	S	161	1298	33		
		173	564 – 734	745 + 712	—	—

(i) Residual glycogen calculated without solubility correction.
(ii) Residual glycogen calculated including solubility correction.
* Shaffer-Hartmann sugar estimation.

same order as the amount which can be accounted for as glycogen when the total carbohydrate and glycogen are determined on the same muscle (Table VII). This is in agreement with the evidence of Table VIII, that the greater part of the lactic acid is formed from glycogen. These experiments do not give sufficient evidence to determine the fate of the carbohydrate not converted into lactic acid.

Laquer [1914, 1921] found that during incubation in phosphate buffer the increase in lactic acid is sometimes greater than the initial carbohydrate content, measured by adding together the glycogen and the lactacidogen. In only two of the results in Table VIII is the initial glycogen content less than the increase in lactic acid, but in several the increase of lactic acid is greater than the decrease in glycogen. This is more often the case with summer frogs than with autumn frogs, and may be a seasonal difference, but both types of result are found in both groups of frogs. The change in alcohol-soluble sugar was only measured in three cases, in all of which it increased, probably owing to the formation of hexosemonophosphate. This increase in alcohol-soluble sugar makes the difference between the decrease of the sum of glycogen and

soluble sugar, and the increase of lactic acid greater where the lactic acid change is already greater than the glycogen change, and more nearly equal, or greater, in the remaining cases, and brings the figures generally more into agreement with those of Laquer. But when the total carbohydrate is measured by acid hydrolysis, as already discussed, the position is quite different (Table IX *a*).

Determinations of residual glycogen, *i.e.* glycogen left in the solid residue after hydrolysis with hydrochloric acid and removal of protein, were carried out on those muscles used for total carbohydrate analysis. The mercuric chloride in the residue does not upset the results, which are of the same order when trichloroacetic acid is used for precipitating protein[1]. In all the muscles where the change in glycogen during incubation was determined a small amount of glycogen was found after incubation. This was approximately the same as the residual glycogen found in the total carbohydrate determinations. It seems possible that this residual glycogen is of a different nature from the main bulk of that present in muscle, since it is not extracted during hydrolysis with hydrochloric acid and is not removed during incubation with phosphate buffer. This raises the question whether or not the correction for solubility in the alcohol used to precipitate the glycogen should be applied in calculating the amount of residual glycogen present, since, if the latter is of a different nature from the rest of the glycogen, it possibly has a different solubility in alcohol. In Table IX the results are shown both with and without the correction; in Table VIII the correction is used in the calculation of all the results.

Bicarbonate buffer. The results of estimations on muscles from autumn frogs of the same batch incubated in bicarbonate buffer are shown in Tables VIII (*d*) and IX (*b*). Although the decrease in total carbohydrate is in all cases except one greater than the increase in lactic acid, the discrepancy is not nearly so great as in the case of incubation in phosphate buffer, and moreover a large amount of carbohydrate is left in the muscle. An increase in hexosephosphate during incubation would tend to make the final figures for total carbohydrate low in comparison with the initial, and possibly this accounts for the greater decrease of total carbohydrate compared with increase of lactic acid. The increase in soluble carbohydrate in Table VIII (*d*) supports the idea of increase in hexosephosphate.

These results and the determination of glycogen and lactic acid agree with those of Meyerhof, of Laquer and of Embden, that lactic acid formation ceases whilst there is still carbohydrate left in the muscle. In two cases in Table VIII (*d*) the lactic acid increase exceeds the carbohydrate decrease calculated from the change in glycogen and alcohol-soluble carbohydrate, though in one it is less than the glycogen change taken alone. If, as is probable, the figures for alcohol-soluble carbohydrate are low, then the change in this fraction should be greater than appears in Table VIII (*d*). This change is always an increase and it is possible that were its full value known it would account for the difference between glycogen increase and lactic acid decrease.

[1] The reducing value after hydrolysis was estimated by the Hagedorn and Jensen method in all cases except two, in which the Shaffer-Hartmann method was used.

As is generally accepted, this difference between the action of the two buffer mixtures suggests that phosphate is required before all the carbohydrate can be converted into lactic acid. When the difference in the behaviour of total carbohydrate is considered it seems probable that in either case the chief source of lactic acid is glycogen, and that the large excess of carbohydrate found when the muscle is extracted with hydrochloric acid, as in this method of determining total carbohydrate, over that found using an alcoholic extraction, cannot form lactic acid at all. It may be noted in this connection that the trisaccharide of Lohmann does not give rise to lactic acid. From a comparison of the action of the two buffer mixtures it seems further very probable that this excess of carbohydrate does not react at all in a bicarbonate buffer, but is converted into a phosphate during incubation in a phosphate buffer. This phosphate may of course only be of a transitory nature.

Changes taking place in muscles poisoned with sodium monoiodoacetate. Lundsgaard [1930] has shown that for muscles poisoned with sodium monoiodoacetate although there is no increase in lactic acid content during anaerobiosis or when the muscle is fatigued, the glycogen content falls and the hexosephosphate content rises proportionately. Lundsgaard has shown that the hexosephosphate is in large part a diphosphate, and from the hydrolysis curve he concludes that it is probably identical with the hexosediphosphate of Harden and Young. This phosphate is much more easily hydrolysed than the monophosphate so that total carbohydrate should show little or no change during anaerobiosis or after fatigue. This was confirmed by experiment, the results of which are shown in Table X. The frogs were injected in the dorsal lymph sac with a 1 % solution of monoiodoacetic acid neutralised to p_H 7 with sodium carbonate. 4 cc. per 100 g. body weight were given. After injection the frogs were placed in the refrigerator for 40 minutes so that the muscles should be in a resting condition when the frog was killed. The muscles were dissected on a board which was well cooled and surrounded by ice so that the changes taking place after death and not due to the treatment given should be a minimum. The control muscles were ground in a mortar with ice-cold alcohol or hydrochloric acid immediately after dissection and weighing. For these experiments it was considered better not to soak the muscles in oxygenated Ringer solution as the changes taking place are not yet fully worked out, and a continuous breakdown might be taking place in the Ringer solution instead of recovery. The muscles were fatigued by single induction shocks at the rate of about 2 per second in an atmosphere of hydrogen. The record of a lever attached to the muscles was taken and in all cases a curve typical of a poisoned muscle was obtained, that is the muscle became inexcitable after 100–200 shocks and remained in a contracted state. For anaerobiosis the muscle was hung in a tube containing hydrogen and either left at 16° for 24 hours, or placed in an incubator at 37° for 2 hours. Determinations were also made of the change in glycogen and alcohol-soluble carbohydrate and sugar in poisoned muscles. The glycogen change is of the same order as that found by Lundsgaard, but the increase in alcohol-soluble carbohydrate does not correspond

Table X.

Change in total carbohydrate, glycogen, alcohol-soluble carbohydrate and sugar, and lactic acid, in muscles poisoned with monoiodoacetate, mg. per 100 g.

Frog	Muscle	Weight	Total carbohydrate initial / final / change	Glycogen initial / final / change	Alc.-sol. carbohydrate initial / final / change	Alcohol-soluble sugar initial / final / change	Lactic acid initial / final / change
			(*a*) Fatigue in hydrogen.				
I	G	1·25	—	1200 964	17 31	6 20	20 22
				964 – 236	31 + 14	20 + 14	22 + 2
II	G	1·10	1260	—	—	—	51
			1237	—	—	—	50
			1237 – 23	—	—	—	50 – 1
III	G	0·77	1115	—	—	—	29
			1115	—	—	—	48
				—	—	—	48 + 19
IV	G	0·90	1082	—	—	—	35
			1085	—	—	—	3
			1085 + 3	—	—	—	3 – 32
V	G	0·44	—	768	27	12	19
				547	57	20	28
				547 – 221	57 + 30	20 + 8	28 + 9
			(*b*) Anaerobiosis in hydrogen.				
I	Gr	0·80	—	1325	42	46	16
				795	92	48	30
				795 – 530	92 + 50	48 + 2	30 + 14
I	Sm	0·30	—	738	43	—	43
				653	103	—	28
				653 – 85	103 + 60	—	28 – 15
II	S	0·20	720	—	—	—	11
			709	—	—	—	30
			709 – 11	—	—	—	30 + 19
II	Gr	1·10	1270	—	—	—	51
			1040	—	—	—	50
			1040 – 230	—	—	—	50 – 1
III	Gr	0·85	—	704	12	11	8
				281	73	84	7
				281 – 423	73 + 61	84 + 73	7 – 1
V	Gr	0·30	878	—	—	—	20
			905	—	—	—	62
			905 + 20	—	—	—	62 + 42
			(*c*) 2 hours in air.				
I	S	0·20	—	670	104	4	37
				590	273	63	78
				590 – 80	273 + 169	63 + 59	78 + 41

with the decrease in glycogen. The values obtained for the former may be low, owing to incomplete extraction of the carbohydrate present by alcohol. In this case the low values cannot be explained by supposing that there is hexosephosphate present which is incompletely hydrolysed, since there is no change in total carbohydrate content, where the time allowed for hydrolysis is less than that allowed in the case of the alcohol-soluble carbohydrate.

Summary.

1. A modification of the method of estimating total carbohydrate of muscle by hydrolysing the whole muscle is described.

2. A modification of Bissinger's method of separating sugar from solution and estimating it quantitatively by precipitation with copper-lime and using the copper as the oxidising agent for sugar estimation is described.

3. The resting value of the total carbohydrate of isolated frog muscles estimated by this method is found to be much greater than the sum of the glycogen and alcohol-soluble carbohydrate.

4. During incubation in a phosphate buffer at p_H 8 this excess carbohydrate is removed from the muscle but does not appear as lactic acid. The lactic acid increase is in general somewhat greater than the decrease of the sum of glycogen and alcohol-soluble carbohydrate.

5. The excess carbohydrate is not removed during incubation in 2 % bicarbonate buffer.

6. In muscles poisoned with monoiodoacetic acid, during anaerobiosis and fatigue the total carbohydrate content does not change significantly whilst the glycogen content falls. The lactic acid content rises very slightly on the average. The alcohol-soluble carbohydrate rises but not sufficiently to account for the fall in glycogen.

7. When the total carbohydrate of muscle is estimated by this method there is left in the muscle a small residual amount of glycogen, on the average 50 mg. per 100 g. muscle. This glycogen does not change significantly in amount during incubation in phosphate buffer. Approximately the same amount of glycogen is found remaining after incubation when the whole glycogen content of the muscle is estimated by Pflüger's method. It is suggested that this glycogen may be of a different nature from the main bulk of that present in muscle.

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